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Physiological Zoölogy

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A CRITICAL EXAMINATION OF WINKLER'S METHOD FOR DETERMINING DISSOLVED OXYGEN IN RESPIRATION STUDIES WITH AQUATIC ANIMALS[1]

W. C. ALLEE AND R. OESTING[1]

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WINKLER'S method of determining the amount of oxygen dissolved in water has been adopted widely as a suitable technique for use in studies on the respiration of aquatic organisms.[2] The method was originally devised for work in quantitative physical chemistry and was used to find the absorption of oxygen in water at different temperatures (Winkler, 1888, 1889). Solubilities determined by this method are given in Hodgman and Lange's *Handbook of Chemistry and Physics*. The method has been much used in studying the oxygen content of natural waters; in fact, Winkler (1888, p. 2851) recommended such use. Alsterberg (1926, p. 70) concludes his critical examination of the usefulness of the method as follows: "Von allen Analysenmethoden für in Wasser gelösten elementaren Sauerstoff ist das von *Winkler* (1888) ausgearbeitete Verfahren am sichersten und einfachsten. Die anderen Methoden sind entweder zu kompliziert für allgemeinen Gebrauch oder ergeben weniger genaue Werte."

The best and most complete review of the literature dealing with the chemical aspects of oxygen determinations by Winkler's method

[1] The tests upon which this report is based were financially supported in part by a grant from the Rockefeller Foundation to aid investigations in the biological sciences at the University of Chicago.

[2] See *Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists* (3d ed. 1930), p. 408.

is given by Theriault (1925). This, together with the earlier review of Elvove (1914) and the later one of Alsterberg (1926) and Theriault and McNamee (1932), makes another such review unnecessary at this time.

Winkler's method has been critically studied in connection with its use as the standard method for determining dissolved oxygen in sewage-contaminated waters, particularly by Theriault, Elvove, and Theriault and McNamee in the citations just given. The standards set for satisfactory results are not as high for such work as they must be for modern studies of respiration. Unfortunately, critical examinations of the usefulness of the method in connection with respiration of aquatic organisms have been few in number. Heilbrunn (1915), in a well-known note, pointed out the possibilities of iodine absorption and discussed methods of overcoming this difficulty. Alsterberg (1926), in a less well-known paper (cf. however, Amberson, 1928), discussed errors arising from the presence of nitrites, iron, hydrogen sulphite, and organic wastes. This paucity of critical evaluation of the method has made possible its dismissal by Shearer (1924) and Parker (1929) with the statement that there are serious objections to its use, without bringing forward or citing concrete supporting data. The present study was undertaken in part to supply this lack, and in part as a necessary preliminary step for determining the proper methodology for studying the respiration of an amphibious animal in water and in air.

ACCURACY OF WINKLER'S METHOD

The usual method of testing the validity of results obtained with Winkler's method has been to compare values obtained by its use with those found when water is boiled and the gases quantitatively determined by standard volumetric methods. Winkler (1888) made such tests. These show that the results obtained by his method, when corrected by us for temperature differences, averaged 0.17 per cent lower than those he obtained by Bunsen's gasometric method. Kisch (1891) repeated these tests and concluded that for the determination of dissolved oxygen in natural and distilled water, Winkler's method is practical, quick, and exact. Even so, of the 11 duplicate determinations, those by Winkler's method averaged 0.92±

0.046 cc. per L. more oxygen than the gasometric method.[3] Comparisons show that the determinations made with Winkler's method on the air-saturated distilled water which Kisch used approach standard values for air saturation at the given temperatures more closely than do the gasometric determinations. In fact, the differences in the former comparisons lack statistical significance while the latter differ significantly from standard values. From this evidence we must conclude that the gasometric determinations of Kisch were in error.

Chlopin (1896) again compared the determination of dissolved oxygen by Winkler's titration method and by Bunsen's gasometric method. For natural waters which contained from 1.0 to 9.98 cc. of oxygen per L., he found a mean difference of 0.21 per cent; in other tests with distilled water the mean difference was about 0.1 per cent.

Birge and Juday (1911) made similar comparisons, using natural lake waters. They report in all on 69 such comparisons with a mean difference of 0.16 ± 0.18 cc. per L. more oxygen by Winkler's method. Shelford and Allee (unpublished) in 1911 again repeated this comparison with similar results.

Oesting (1934) tested determinations by this method against those obtained by a modified Van Slyke method using an artificial-pond water. In part he used a nitrite-free water and Winkler's unmodified method and in part a nitrite-containing water and the permanganate modification originally suggested by Rideal and Stewart (1901). These data are shown in detail in Table III of his paper and in Table II of the present report. Combining all his data, there is a difference of 0.018 ± 0.01 cc. per L. more oxygen given by the modified method of Van Slyke. This mean difference has a statistical probability of 0.072; since 0.05 is usually taken as the upper limit of statistical significance, this difference is not statistically significant.

These comparisons, together with others to be discussed later, are summarized in Table I in terms of the mean percentage of difference between the determinations by Winkler's and by other methods. It should be noted that while Birge and Juday (1911) report differences for 69 comparisons, they give complete data from which percentage

[3] Standard errors are shown unless stated to the contrary.

differences can be calculated for only 24 of these. The mean differences in dissolved oxygen determinations reported by five different observers range from +0.6 to −0.77 per cent, with a mean of −0.1 ±0.16 per cent. This would indicate a maximum mean error in these tests of approximately 0.016 cc. per L. with air-saturated water at 20° (see, however, p. 513 and p. 522). Elvove (1914, p. 31) regards 0.04 cc. per L. (0.06 p.p.m.) as lying within the limit of the experimental error of the ordinary Winkler method.

TABLE I

PERCENTAGE DIFFERENCES BETWEEN DISSOLVED OXYGEN AS DETERMINED BY WINKLER'S AND BY GASOMETRIC METHODS

Observer	Mean Difference in Per Cent	Comments
Winkler	−0.17	Distilled water
Chlopin	+0.21	Natural water
	+0.1	Distilled water
Birge and Juday	−0.13	Wisconsin lake water
Letts and Richards	+0.6	Rideal-Stewart-Winkler, tap water
	−0.77	Rideal-Stewart-Winkler, sea water
Oesting	−0.34	Artificial pond water
Mean	−0.1 ±0.16	

Another check deserves mention. Whipple and Whipple (1911) were engaged in finding the solubility of oxygen in sea water and in brackish waters and were using Winkler's method when the work of Fox (1909) came to their attention. Fox had covered the ground they were exploring, using a modified form of Estreicher's adaptation of Oswald's method and had obtained results which Whipple and Whipple state "agreed with our own, as far as they had been carried." No precise data are given from which percentage of variation can be calculated.

Theriault (1925, p. 16) concludes a critical examination of the chemical literature dealing with Winkler's method as follows: "When ordinary analytical safeguards are practised and when interfering substances are absent the permissible error with the original Winkler

method should not exceed a few hundredths of a part per million."[4] Estimating this in another manner, with water saturated at 20°, an error of 1 per cent would amount to an error of approximately 0.09 p.p.m. Theriault (1925) found an average loss of from 0.04 to 0.15 p.p.m. when the sample is poured out for titration in the usual manner as compared with the removal of a standard amount and titration of the remainder directly into the sampling bottle. If an error of this magnitude is inherent in the titration method as usually practiced, it is difficult to understand the close agreement found with the gasometric methods such as has been summarized in Table I. Increasing the amount of potassium iodide would decrease the iodine loss through volatilization at pouring.

Another approach to the question of the value of Winkler's method for work on respiration is to examine its repeatability. In such work one disregards the absolute error and focuses on the relative error. Winkler gives data of two sorts which allow this comparison. He determined the amount of oxygen dissolved in air-saturated distilled water at four different temperatures and compared the observed values with the calculated air saturation at those different temperatures and found percentage differences of +0.07, +0.20, −0.11, and +0.63 per cent. The mean error is approximately +0.2 ±0.16 per cent.

In his 1889 paper he reports the details of 70 determinations made at different temperatures from +0.11° to 14.8° with two or usually three determinations of the oxygen content of air-saturated distilled water at each temperature. The average departure from the 26 given means is 0.0054±0.0004 cc. per L. or an approximate deviation from the mean of 0.07 per cent. Many other such comparisons could be given. Allee and Fowler (1932) report that 10 successive tests of the oxygen dissolved in the same body of sea water made with considerable care showed a mean of 5.30±0.009 cc. per L. or an error of 0.34 per cent. Similar tests on a different body of water, using the less rigorous procedure characteristic of a long series of routine determinations, showed a mean of 5.67±0.036 cc. per L. The error here is 1.27 per cent.

[4] To change parts per million to cc. of oxygen per L. multiply by 0.6978.

THE NITRITE ERROR

Winkler's original paper (1888, p. 2852) showed an appreciation of the error introduced by the presence of nitrites. If more than 0.1 mg. of nitrous acid per L. of water was present, he recommended special treatment to oxidize the nitrite to the non-interfering nitrate; otherwise too high a value was obtained. Without discussing all the other modifications suggested by various workers (see Elvove, 1914, and Alsterberg, 1926), Rideal and Stewart (1901) produced a less cumbersome modification based on the same principle but using different reagents. An improved form of this permanganate modification has been adopted for nitrite and sewage-contaminated waters. The amount of oxygen dissolved in waters was determined by this method and by standard gasometric analyses by Letts and Richards for the British Commission on Sewage Disposal (1913). One per cent city sewage mixed with (*a*) tap water and (*b*) sea water and incubated from 5 to 42 days at different temperatures yielded a series of dissolved oxygen values from 6.2 to 0.2 cc. per L. The 12 pairs of determinations of the oxygen content of the sewage contaminated tap water showed a mean difference between titration and gasometric determinations of $+0.025 \pm 0.045$ cc. per L. Twelve similar pairs of determinations in sea water gave a mean difference of -0.025 ± 0.052 cc. per L.

Alsterberg (1926, p. 47) reports experiences with the permanganate modification as compared with Winkler's original method. He used air-saturated distilled water but does not state at what temperature. The unmodified Winkler's method gave 4.63 and 4.61 cc. per L. The results with the modification of Rideal and Stewart were 4.58 and 4.59; a mean difference of 0.035 cc. per L. and a percentage difference of 0.75. Alsterberg concludes from these data, which are all that he gives on this point, that the permanganate modification is not satisfactory, and after discussing the merits and demerits of other modifications proposes his own, which will be discussed later.

Tests reported by Elvove (1914, p. 30) show the same tendency toward a small but consistently lower value with the permanganate modification. He used water with 8.87 p.p.m. dissolved oxygen, added 5.0 p.p.m. of nitrite nitrogen, and found a mean of 8.81 ± 0.008 p.p.m. of oxygen with a mean error of -0.06 ± 0.007 p.p.m. This

difference, while low, and, as Elvove believes (p. 31), "within the limit of error of the ordinary Winkler method," has a statistical probability of 0.00028 and from the statistical point of view is highly significant. Clark (1929, p. 56) states that in his experience the permanganate modification tends to give low values with ordinary waters. Noll (1917) tested various kinds of water to which nitrites were added and found a mean departure of -0.0024 ± 0.0108 cc. oxygen per L. from the determinations on nitrite-free water made by Winkler's unmodified method. This is approximately 0.07 per cent variation from the mean of all the samples tested.

We have examined again the extent of the nitrite error, using a nitrite-free, artificial pond water made up as follows: $CaCl_2$, 200 mg.; K_2SO_4, 100 mg.; $NaNO_3$, 100 mg.; and $MgSO_4$, 100 mg. per L. of distilled water. The amount of nitrite (in terms of mg. per L. of nitrite nitrogen) added to this water varied in different tests between 0.1 and 0.005 mg. per L. The water was approximately air-saturated at 25°, and was made up four different times, as shown by the sections of Table II. The data given in this table are important in that they show a nitrite error with Winkler's unmodified method when there is as little as 0.01 mg. of nitrite nitrogen per L. and further because of the detailed check between the permanganate modification and Van Slyke's method. It is important also to note that the end point in this and other work with Winkler's method reported for the first time in this paper was taken to be the point when no trace of blue color remained after one minute of steady stirring after adding the final drop of sodium thiosulphate.

The mean difference between determinations by the permanganate modification of the Winkler method and by Van Slyke's methods is seen to have been 0.005 ± 0.007 cc. per L. more oxygen by the former. The statistical probability is 0.49 and the percentage of error is 0.09. For the conditions tested, the two methods check each other remarkably well. The nitrite error as shown by the differences between columns II and III is analyzed in Table III.

The lack of statistical significance at 0.025 and 0.05 mg. nitrite nitrogen per L. is due to an unusually high value obtained with Winkler's unmodified test in one determination each. Such erratic determinations illustrate the difficulty of establishing an exact end

point in the presence of nitrites. Despite this lack of statistical significance, the statistical probability obtained with 0.01 and with 0.005 mg. nitrite nitrogen indicates that the latter must be taken as near the maximum nitrite that can be tolerated with the unmodified method. This marks a decided reduction in tolerance from the 0.1

TABLE II

DETERMINATIONS OF DISSOLVED OXYGEN BY WINKLER'S UNMODIFIED METHOD; BY RIDEAL AND STEWART'S MODIFICATION AND BY VAN SLYKE'S METHOD WHEN SMALL AMOUNTS OF NITRITE ARE PRESENT; THE NITRITE IS EXPRESSED IN MG. PER L. OF NITRITE NITROGEN

(Dissolved oxygen cc. per L.)

I Nitrite Content; Mg. N_2 per L.	II Winkler Unmodified	III Rideal-Stewart-Winkler	IV Van Slyke	III–IV
0.01	6.00	5.84	5.81	0.03
0.025	5.87	5.77	5.80	−0.03
0.05	6.03	5.80	5.84	0.04
0.075	6.23	5.85	5.84	0.01
0.10	6.53	5.87	5.83	0.04
0.005	5.90	5.85	5.84	0.01
0.005	5.71	5.71	5.70	0.01
0.01	5.80	5.69	5.68	0.01
0.05	6.05	5.77	5.75	0.02
0.10	6.33	5.75	5.76	−0.01
0.50	7.71	5.79	5.76	−0.03
0.005	5.79	5.75	5.74	0.01
0.01	5.83	5.72	5.74	−0.02
0.025	5.92	5.75	5.75	0.00
0.05	6.03	5.78	5.76	0.02
0.10	6.33	5.77	5.76	0.01
0.005	5.83	5.80	5.79	0.01
0.01	6.10	5.78	5.79	−0.01
0.05	6.23	5.82	5.80	0.02
0.10	7.16	5.71	5.82	−0.11
0.50	8.51	5.89	5.83	0.06
0.25	7.81	5.82	5.80	0.02

nitrite nitrogen[5] per liter allowed by the *Official and Tentative Methods of Analysis* already cited. The decrease in toleration may be due, in part, to our use as an end point the failure to return of blue color after one minute of stirring rather than the initial disappear-

[5] Winkler (1888, p. 2852) stated the toleration limit of his unmodified method as 0.1 mg. "salpetrige Säure" per L. Alsterberg (1925, p. 36) repeats this limit and interprets "salpetrige Säure" as N_2O_3. The toleration established by Winkler and by Alsterberg, while greater than our own, is decidedly less than that used in *Official and Tentative Methods*.

ance of blue color as is apparently intended by the instructions given in the analytical methods just cited. Our end point is at once more constant and more sensitive.

The permanganate modification causes the introduction of a larger volume of reagents than is necessary in the unmodified method. These reagents presumably are at air saturation as regards dissolved oxygen. When they are in concentrated form and when a comparatively large volume of water is used as a sample, correction for the additions is small and can usually be neglected. When, however, the oxygen content is low, the reagents, being presumably air saturated with oxygen, will make the result too high. Rideal and Stewart (1901) give a formula for such a correction which is, however, not needed in the usual respiration studies since the same amount, presumably, would be added to all the samples tested.

TABLE III

THE NITRITE ERROR WITH DIFFERENT CONCENTRATIONS OF NITRITE NITROGEN; A SUMMARY OF TABLE II

Nitrite in Mg. of N_2 per L.	Difference between Winkler and Rideal-Stewart-Winkler Determinations	Statistical Probability
0.005	0.03	0.068
0.01	0.175	0.0398
0.025	0.75	0.3550
0.05	0.85	0.2702
0.10	0.84	0.028

The effect of this nitrite error on the results obtained in recent studies on respiration will be discussed later in this report. In view of the low nitrite nitrogen content which affects the determination of dissolved oxygen by Winkler's unmodified method, and further in view of the fact that the presence of nitrites renders the precise determination of an end point more uncertain with this method, it should be made routine procedure in all studies on respiration to establish the absence of troublesome amounts of nitrites[6] at the end of

[6] For methods see "Official and tentative methods of Analysis." Assoc. Official Agric. Chemists. 3rd Ed. p. 405. Hellige furnishes standard color disks for nitrite determination.

the respiration period unless the permanganate modification or some other equally satisfactory one is used at all times.

THE ERROR FROM IRON SALTS

Rideal and Stewart (1901) report that even in the presence of organic matter and ferrous iron, their permanganate treatment gave results "almost identical with gasometric figures." Theriault (1931) estimates that 1 p.p.m. of ferrous iron will occasion an apparent loss of oxygen of about 0.14 p.p.m. with the ordinary method of Winkler. There is no doubt but that treatment with permanganate will oxidize ferrous iron to ferric, but the presence of the latter produces a need for added precautions. If iron salts are present, Theriault (1925, p. 39) concludes that accurate results with water containing as much as 100 parts of iron salts per million can be obtained with the permanganate modification providing the following precautions are taken; "(*a*) The decolorization should proceed in the dark. (*b*) The samples should be practically colorless when the Winkler reagents are added. (*c*) The titrations should be performed immediately after the final acidification."

Alsterberg (1926, p. 60) calculates on the basis of experimental experience with larger amounts of ferrous iron that 5 mg. per L., a quantity which has been reported from natural waters, would cause an error of from 0.4 to 0.5 cc. oxygen per L. if Winkler's unmodified method is used. This would amount to an error of from 8 to 10 per cent with oxygen saturation at ordinary temperatures. Since he regarded the permanganate modification as unsatisfactory, he proposed preliminary treatment of iron-contaminated waters with bromine and subsequent removal of the excess bromine with salicylic acid. This would be followed by treatment with the usual reagents of Winkler's method except that phosphoric (H_3PO_4) rather than sulphuric acid is used for the final acidification.

In preliminary tests with air-saturated distilled water (p. 58) Alsterberg reports that results with the unmodified Winkler's method were 5.29 ± 0.0058; those with the bromine modification were 5.30 ± 0.000. In the presence of about 40 mg. per L. of ferrous iron, the unmodified method showed an error of approximately 72 per cent while the bromine modification yielded only 0.2 per cent error.

Alsterberg found no error whatever with ferric iron either with or without the bromine modification.

It is clear that in order to obtain exact values with Winkler's unmodified method, the water to be tested together with the reagents to be added must contain only a fraction of a milligram of ferrous iron per L. and that in respiration studies care must be taken that the respiring material does not increase the amount of such iron present. If ferrous iron is present, one of these preoxidation techniques should be used and precautions should be taken, if necessary, to avoid possible interference by ferric salts.

THE ERROR FROM IODINE ABSORPTION

Heilbrunn (1915) commended Winkler's method for its "accuracy and convenience" but pointed out that water in which sea-urchin eggs had stood contained iodine-absorbing powers which affected such determinations even if Rideal and Stewart's modification was used. He suggested among other remedies that the iodine-absorbing power of supposedly fair samples should be ascertained and correction made. As he stated, the difficulty here lies in the validity of the sampling.

Shelford and associates (1934) record that in Puget Sound the amount of dissolved oxygen near the bottom is generally very low and the iodine absorption is so great that the small amount of iodine liberated is soon absorbed, perhaps immediately. Under these conditions Winkler's method is useless.

Hyman (1916, p. 242) tested the iodine absorption of surface sea water of Puget Sound in connection with experiments on respiration of sponges carried on at the Puget Sound Biological Station. She found this value "so extremely small as to be entirely negligible"; no figures are given. Later (1919, p. 362) she tested the iodine-absorbing power of Hull Zoölogical Laboratory well water (University of Chicago) and found it to be appreciably less after planarians had lived therein. As will appear later, such results suggest the presence of nitrites. Again (1932*a*) Hyman tested the iodine absorption of water in which planarians or *Nereis* had lived accompanied with that of untreated and of distilled water. Nine paired determinations of water from planarian worms compared with control water showed a

mean difference of 0.003±0.018 cc. more of N/10 sodium thiosulphate required to reduce the iodine in the worm-conditioned than in the untreated control water. Distilled water absorbed slightly more iodine than did the worm-conditioned water, while sea water containing *Nereis* absorbed slightly less iodine than did a similar amount of untreated sea water; there is no statistical significance in these results.

Lund (1918) tested the oxygen consumption of *Paramecia* by Winkler's method. He added the reagents directly to the bottles which contained the infusorians and by appropriate checks determined the amount of the liberated iodine adsorbed by the dead organisms. The adsorbed iodine varies with the number of *Paramecia* present, the concentration of iodine and temperature. In his experiments the equivalent of from 0.23 to 0.3 cc. of oxygen per L. was removed by these animals.

We have recently tested the iodine adsorption and absorption error in a liter of well water in which four goldfishes 40–50 mm. long had lived for 30 hours and found it to be equivalent to less than 0.023 cc. of oxygen per L. This is approximately 0.37 per cent of the oxygen present and indicates the iodine error in fairly heavily fish-conditioned water. Enough has been said to indicate that in all future use of Winkler's method in respiration studies, the water being analyzed must be tested for iodine absorption at a pH near that encountered after the final addition of acid in Winkler's method, and corrections made if necessary.

OTHER ERRORS

While the permanganate modification of Winkler's original method is useful in dealing with water containing nitrites and ferrous salts and the forms of organic matter commonly present in freshly aerated sewage, it is not suitable for use in waters containing large amounts of dextrose, peptone, or sulphite wastes or in the presence of stale sewage (Theriault and McNamee, 1933). If as little as 10 p.p.m. of glucose is present, there is an appreciable decrease in oxygen revealed by the unmodified or by the permanganate modification of Winkler's method; this is said to be less pronounced with the permanganate modification. The error can be practically eliminated,

according to Theriault and McNamee, even in the presence of as much as 400 p.p.m. of glucose if the period of alkalinization is decreased to 25 or to 15 seconds, and the sample is shaken constantly during that time. With 5,000 p.p.m. of glucose, the apparent loss with short alkalinization is reported to be 0.51–0.37 cc. of oxygen per L. It appears from the work of Clark (1929) that ten minutes is a minimum period for alkalinization of sea water, hence this procedure is inapplicable there. Theriault and McNamee maintain that fifteen to twenty-five seconds is sufficient for sewage-carrying river waters when there is 1 ml. of each of Winkler's reagents per 150 cc. sample; and this despite the fact that Theriault (1925, p. 19) showed analyses which indicate that in order to secure the most accurate results with the usual settling procedure the manganic hydroxide precipitate should be allowed to settle, then reshaken and allowed to settle again.

In the presence of peptone, work of the men just cited shows that the unmodified method is preferable to the permanganate modification. Abbreviated alkalinization adds to the accuracy. With 120 p.p.m. of peptone, the apparent loss of oxygen was 0.81 cc. per L. by the permanganate modification; 0.21 by Winkler's unmodified method; and 0.1 cc. per L. by the latter method with 25 seconds' exposure to alkali.

If sulphite wastes are present even in fairly large amounts, the oxygen content can be determined with the same degree of accuracy by use of an alkaline-hypochlorite modification of Winkler's method which has been described and tested by Theriault and McNamee (1932).

Cyanides interfere with determinations by Winkler's method and precautions must be taken if a cyanide is present in the solution undergoing analysis. Hyman (1919) made this correction by putting the same amount of cyanide in both the experimental and the blank flasks. This should yield reliable results providing the respiring organisms do not remove an appreciable amount of the cyanide from solution. There is indirect evidence from the work of Hyman (1916, 1919, 1919a) and of Lund (1918) that the cyanide as used in their experiments did not affect their oxygen determinations significantly; Hyman[7] has unpublished data which demonstrate the lack of cyanide

[7] Personal communication.

error with the concentrations of cyanide used. Alsterberg (1926) gives data showing that ferrocyanide in amounts sufficient to cause an error of 7.9 per cent with Winkler's unmodified method, if treated with his bromine modification, causes an error of only 0.2 per cent. This modification appears useful also with larger amounts of ferricyanide; smaller amounts of the latter substance (2.5 mg. per 125 cc.) did not interfere with determinations by the unmodified method.

An excess of oxalate used in the permanganate modification introduces an error which can be overcome by adding more than the usual amount of KI (Elvove, 1914). Theriault (1925, p. 26) estimates that with proper control oxalate errors should not be greater than 0.1 p.p.m. An excess of acid causes a slow evolution of iodine from the KI (Lund, 1921). As ordinarily used, however, and for medium to large values of dissolved oxygen, the error from allowing samples to stand up to 48 hours after the addition of the final acid is not appreciable (Birge and Juday, 1911). The keeping qualities for this length of time are not affected by the presence of the excess of oxalic acid which may remain after Rideal and Stewart's modification has been used.

Another element of weakness in the use of Winkler's method in measuring the rate of oxygen consumption has been the frequent failure to secure large enough differences between initial and final determinations, or between those in the control and those in the respiration chambers, to allow for the error inherent in the method itself. Lund (1921) estimated that as generally used this error amounts to about 1 per cent, which would be about 0.05 cc. per L. Elvove (1914) thought that 0.04 cc. per L. lay within the experimental limit of error. It has been our experience that with usual contents of oxygen we can be sure of repeatability to about 0.03 cc. per L. All these estimates are in the same general field of values. Hyman's[8] error as determined by duplicate determinations was 0.02 cc. per L. When the animals being tested consume 2.0 cc. of oxygen per L., the error inherent in the method amounts to from 1.0 to 2.5 per cent; this is allowable. When, however, small differences are recorded, the inherent percentage of error mounts.

If in addition there is an error from uncontrolled movement such as occurs with most aquatic organisms, or individual variability for

[8] Personal communication.

any other cause, this also must be considered and the amount of oxygen consumed should be arranged so that the combined error to be expected from these two uncontrolled factors should not exceed 5 or 6 per cent of the observed difference. Fortunately it is possible with some organisms to control movement; planarians with their heads recently removed remain quiet in the respiration chamber (Hyman, 1919). Etkin (1934) found that the uncontrolled movement of tadpoles introduced an error of approximately 4 per cent.

MICRO-WINKLER METHODS

Lund (1921) in a brief note described a so-called micro-Winkler method suitable for 10 or even for 5 cc. samples. He used 0.1 cc. each of the $MnCl_2$ and $NaOH+KI$ solutions and titrated with a thiosulphate solution diluted to one-tenth its usual strength. The inherent error was stated to be about the same as in the ordinary Winkler method as used by the author, namely, about 1 per cent. Winkler (1924) applied his unmodified method, and in the presence of nitrites his hypochloride modification (Winkler, 1915), to samples of 24–30 cc. of water by suitably modifying the solutions required. Thompson and Miller (1928) and Kawaguti (1933) have described special pieces of apparatus designed to make such determinations with as little as 0.1 cc. of water (Kawaguti) without air contamination in sampling or at the time of introduction of reagents. Comparisons by Snoke (1929) using Thompson and Miller's apparatus with 4.9 cc. samples and the usual Winkler's method with 250 cc. samples showed a mean of 0.003 of the units used (probably p.p.m.) less oxygen by the micro-method. Kawaguti (1933) reports a steadily increasing decrease in the amount of oxygen in successively smaller samples of air-saturated distilled water. He regards this as correlated with the insensibility of the iodine-starch reaction.

One of us (Allee, 1929) used a modification similar to that described by Lund except that the samples approximated 14 cc. In 50 determinations summarized in ten means, the average departure of any determination from its mean was 0.0037 ± 0.0007 cc. per L., or approximately 0.23 per cent. This would indicate that the micro-Winkler method as used had about the accuracy of the regular method.

So far as we have found, there have been no reports hitherto of

the accuracy of a so-called micro-method for the permanganate modification of Winkler's method. In the tests we have to report, samples of from 116.9 to 120.4 cc. were analyzed by the usual permanganate modification and parallel samples of from 14.4 to 15.8 cc. were analyzed, using 0.1 cc. of the usual reagents except that the initial sulphuric acid used was diluted in half. The final sulphuric acid was full strength as usual. The results obtained are shown in Table IV. Here and elsewhere where the permanganate modification has been used in the present studies, the permanganate was allowed to act for 40 minutes rather than for the 20 minutes usually practiced.

TABLE IV

COMPARING DETERMINATIONS OF DISSOLVED OXYGEN MADE WITH THE PERMANGANATE MODIFICATION (RIDEAL-STEWART) OF WINKLER'S METHOD, USING RELATIVELY LARGE AND RELATIVELY SMALL SAMPLES; ALL DETERMINATIONS SHOWN ARE AVERAGES FOR SETS OF THREE

Sample	116.9–120.4 Cc.	14.4–15.8 Cc.	Differences
1	4.963	4.956	0.007
2	5.323	5.330	−0.007
3	5.276	5.296	−0.020
4	5.710	5.743	−0.033
5	5.653	5.670	−0.017
6	5.746	5.733	0.013
7	4.976	4.983	−0.007
8	5.676	5.696	−0.020
9	5.743	5.720	0.023
10	4.930	4.930	0.000
Mean	5.400	5.406	−0.006

There is a statistical probability of 0.276 of the mean difference being significant; this means that the percentage difference of 0.11 per cent is far from having statistical significance and that the smaller volumes may be used when needed in place of the larger. With the smaller volumes, great care must be taken to know the normality of the dilute sodium thiosulphate solution used to titrate them.

MINERAL OIL AS A PROTECTION FROM OXYGEN CONTAMINATION

It has been standard practice for some time to cover water used in respiration studies with a layer of oil to avoid oxygen contamination

from the air. Paraffin oil, despite the favorable experience of Amberson, Mayerson, and Scott (1924), has been shown to allow oxygen to pass fairly readily (Hill, 1928; Lallemand, 1932). Heavier oils have usually given satisfaction (Helff, 1928; Allee and Fowler, 1932; Bowen, 1932; and Schuett, 1933). Since, however, Kubie (1927) found the solubility of oxygen in mineral oil at 24° to be 0.134± 0.004,[9] while the solubility of oxygen in water is 0.02881 at that temperature, it seemed desirable to test again the efficiency of a mineral-oil seal such as had been used in the studies just cited. Upjohn's *White Mineral Oil* as obtained at local drug stores was the oil used. Goldfishes were allowed to reduce the oxygen content of an air-saturated artificial pond water either covered with oil, method I, or in a stoppered bottle, method II, until there was less than 0.2 cc. per L. oxygen present. In method II the rubber stopper was removed and the water covered with oil when the desired oxygen content had been established. Water was removed through a side outlet near the bottom to allow the standard oil layer to be introduced. In both methods the fishes were removed through the oil by means of a small net with the minimum possible agitation. The volume used was approximately 2500 cc. with a surface area of 68.3 cm^2. The oxygen content was determined immediately after the removal of the fishes and after an interval of 24 hours with results as shown in Table V. Tests were made at room temperatures which ran as high as 26°.

When method II was used and when there was only 1 cm. of oil used with method I, significant leakage occurred in 24 hours (mean 0.0145±0.0037). When, however, 2–5 cm. of oil was used and the water treated as in method I, no detectable change occurred in 24 hours.

At times water is boiled to drive off contained gases and after partial cooling is covered with oil as a protection against air contamination. The efficacy of such oil seals was tested in part as follows: Twelve flasks were filled with 2 L. each of artificial pond water and covered with 5 cm. of oil. The oxygen content was reduced as in method I. After removal of the fishes, the flasks were allowed to stand overnight and then were heated while still sealed with oil to 50° for 2 hours, 60° for 2 hours, and 50° for 4 hours. Adequate un-

[9] Presumably the probable, not the standard, error.

heated controls showed no oxygen leakage. Duplicate analyses of the three conditions tested showed a mean oxygen leakage of 1.006± 0.05 cc. per L. during the time from the application of heat until the vessels were again at room temperature. It is probable that the heated oil is a less efficient seal because of its loss of viscosity with heat and the resulting increase in convection currents (cf. Hill, 1929).

TABLE V

TESTS FOR OXYGEN LEAKAGE THROUGH A SEAL OF HEAVY MINERAL OIL

Cm. of Oil	O_2 when Fishes Were Removed; Cc. per L.	O_2 after 24 Hours; 22 per L.	Oxygen Leakage	Method
5	0.152	0.152	0.0	I
2	0.095	0.095	0.0	.....
5	0.087	0.087	0.0	.....
2	0.113	0.113	0.0	.....
5	0.108	0.130	0.022	II
2	0.134	0.144	0.010	.....
1	0.075	0.097	0.022	I
1	0.107	0.107	0.0	.....
1	0.107	0.129	0.022	.....
1	0.129	0.150	0.021	.....
5	0.152	0.152	0.0	I
5	0.108	0.108	0.0	.....
5	0.096	0.096	0.0	.....
				.....

DISCUSSION

The use of Winkler's data as the basis for the table on the solubility of oxygen in water by the editors of the *Handbook of Chemistry and Physics* indicates that the method will yield reliable data with distilled water. The consistency of the results obtained by its use in studies of the respiration of various aquatic organisms and the closeness of the checks between data obtained by this method and that obtained by other methods indicate that it is useful in such studies. The strong commendation of the method by Alsterberg (1926) which has been quoted is to be taken more seriously since it comes from the most extensive and critical review recently made of the applicability

of Winkler's method to biological, as distinguished from sewage problems, and further because it was made by a person with a background of previous experience with respiration studies on sewage-inhabiting tubificid worms. Our own conclusions based on previous experience of Allee and his associates and on the direct tests reported here for the first time are that under proper conditions and with appropriate technical care results on respiration of aquatic organisms obtained by the use of Winkler's method or of certain of its modifications have high relative value and approach good absolute values.

With animals such as tadpoles, which show muscular movement in the respiration chamber, the method is much more dependable than is needed; thus Etkin (1934) found that the mean deviation of a series of blanks from the average of paired determinations amounted to 0.3 per cent with a standard deviation of 0.26 per cent. The error due to the uncontrolled movements of the tadpoles, whose respiration he was studying, amounted to 4.0 per cent with a standard deviation of 4.2 per cent. With such motile animals any method which requires shaking of the respiration chamber in order to establish equilibrium with overlying gases which will then be analyzed must, as Hyman (1932) correctly states, greatly increase the error due to muscular activity. Placing animals in small retaining chambers and so suppressing obvious movements may or may not decrease muscular activity. Placing aquatic organisms in an atmosphere of moist air or in a very small surrounding drop of water must be experimentally justified before results can be taken to represent the rate of respiration of the organism in question when surrounded by its usual medium. For that matter the placing of an animal in the artificial bareness of the respiration chambers used in the usual tests made with Winkler's method may introduce fundamental errors if one is trying to find the respiration relations of animals under natural conditions (cf. Hyman, 1932).

The intrinsic methodological demands made by respiration studies differ from those of physical chemistry, on the one hand, or of sewage engineers, on the other. For many, though not for all, purposes the student of respiration is less concerned over the exact amount of oxygen present at the beginning and at the end of a respiration period than he is with the exact amount removed during that period.

Thus errors of fact can be tolerated if there is assurance that the absolute, not the relative, error is the same for samples taken at the two ends of the respiration period or in the sample from the respiratory chamber and the accompanying blank.

The troublesome sources of error in the use of Winkler's method in work on respiration lie in the possibility of contamination of the water by the respiring organisms with materials which affect the accuracy of the determination. The safeguards to be taken, beyond the usual care necessary for any quantitative chemical determination, lie in ascertaining whether such contaminations have increased beyond the limits of analytical tolerance and, if so, in introducing appropriate modifications which will yield results in the presence of troublesome contaminations. Until adequately rechecked, all work done in the absence of such precautions must remain under suspicion. There is, however, no need to struggle for exactness of method of determination when uncontrolled movements introduce errors greater than those inherent in the method of analysis used.

We have already shown that, as used in respiration studies at the University of Chicago, the possible error from iodine absorption or adsorption (Heilbrunn, 1915) is usually negligible and has been controlled adequately. The matter that calls for detailed discussion is concerned with the effect of the possible presence of nitrites and of ferrous iron on determinations with the unmodified method of Winkler. Of these, we have not investigated ferrous iron.

It is not within the limits of our program to check adequately all the work on respiration which has been done by the unmodified Winkler's method. We are concerned primarily in testing the limits of accuracy of work done by Allee and his associates upon the relation between numbers present and the rate of oxygen consumption.

PLANARIAN RESPIRATION

On account of the prominence of the work and in view of certain recent criticisms (Shearer, 1926, 1930; Parker, 1929), we have investigated to a slight extent the possible existence of a nitrite error in the work of Hyman on planarian respiration (1919, 1919*a*, 1920, 1923). Wherever we have made direct tests we have either failed to find nitrates above our revised limit of tolerance, or, if present,

sharpness of end point aside, they need not have affected the specific experimental results we were testing. We have not, however, made an adequate rechecking of all of her extensive respiration studies, and we have evidence that under certain conditions approaching those found in some of her experiments planarians may produce nitrites directly or indirectly in quantities sufficient to affect results obtained with Winkler's unmodified method, which was the one Hyman used in all of her studies.

Specifically, in one test, we made conditions as nearly like those described by Hyman (1920, p. 406) in her tests of the effect of starvation on rate of oxygen consumption of planarians as we could readily do except that we used aliquot parts of water and of planarians. There is the further difference that she used *Euplanaria maculata* for which we substituted *Euplanaria dorotocephala*, the species Hyman had used in similar studies in 1919. Hyman made her tests in Hull Zoölogical Laboratory well water, which we have tested and found in 1933 to be free from nitrites. She used two hundred worms in 500 cc. of water and made the oxygen determinations after 2 hours of respiration (1920, p. 417). We placed forty worms in 100 cc. of Hull well water which was nitrate free and then tested for nitrites after 2 hours. The worms were 10–12 mm. long, one of the sizes used by Hyman; our tests were made at 20°, the temperature she had used and on worms which had starved 15 days which corresponded to one phase of her tests. One set of worms had been decapitated on the day preceding the test and so were like those used in one series by Hyman. The quantities of nitrites produced by the worms we tested are shown in Table VI.

Data presented elsewhere indicate that in three of each set of four determinations nitrites were produced in sufficient amounts to have caused an appreciable error in oxygen determination. Adjustment for this nitrite error does not affect her conclusion (p. 414) that "the rate of oxygen consumption per unit weight is increased during starvation" unless one makes the assumption that there is a decreasing nitrite error with increasing starvation and that the resulting increase in apparent oxygen consumption is in reality an expression of the decrease in nitrites. Regarding the possibility of this assumption being true, we have no evidence.

We have also checked Hyman's work on pieces of *Euplanaria dorotocephala* (Hyman, 1923) to see if such pieces produce nitrites in significant amounts. In a part of this work Hyman tested the rate of oxygen consumption in decapitated worms with the posterior tips

TABLE VI

TESTS FOR POSSIBLE NITRITE ERROR WHICH MIGHT AFFECT THE RESULTS OBTAINED BY HYMAN IN PLANARIAN RESPIRATION (HYMAN, 1920, p. 406); NITRITES ARE RECORDED IN MG. OF NITROGEN PER L.

Intact Animals		Heads Removed for 24 Hours	
Test No.	Nitrite Nitrogen	Test No.	Nitrite Nitrogen
1	0.009	1	0.009
2	0.0075	2	0.009
3	0.01	3	Trace
4	Trace	4	0.01

also removed, so-called "whole" worms, as compared with similarly treated planarians which were then cut into eight pieces each (p. 50). Each lot in Hyman's experiments consisted of twenty-five to thirty worms, which had been starved for 6–9 days; apparently they were placed in 500 cc. of water at 20° for 5 hours immediately after section.

TABLE VII

TEST FOR POSSIBLE NITRITE ERROR WHICH MIGHT AFFECT RESULTS OBTAINED BY HYMAN IN RESPIRATION OF PLANARIAN PIECES (HYMAN, 1923); NITRITES ARE RECORDED IN MG. PER L.

Test No.	Nitrite Nitrogen	Test No.	Nitrite Nitrogen
1	0.0000	3	0.0002
2	0.0001	4	0.0000

In our tests we took six worms of this species and length and starved them for 16 days, cut off their heads and tails, and then cut each into eighths. They were immediately placed in 100 cc. of nitrite-free water. The tests for nitrite nitrogen are summarized in Table VII and show no appreciable increase in nitrites.

Hyman (1923, Table I, p. 51) compared the rate of oxygen con-

sumption of "whole" worms with that of thirds and of eighths; she found the lowest rate of oxygen consumption given by the "whole" animals. The results given in our Table VI shows that headless worms may produce nitrites in 2 hours sufficient to cause difficulty with Winkler's unmodified method. Comparing these data with those just given in Table VII, a greater nitrite error for the whole worm would be indicated than for the worms divided into eighths. This would lessen the differences Hyman found to exist between them in the matter of oxygen consumption.

Such comparisons are unsatisfactory when direct ones can be made. Accordingly this experiment was repeated, using "whole"

TABLE VIII

NITRITES PRODUCED BY "WHOLE" PLANARIANS AND BY SIMILAR WORMS CUT INTO EIGHTHS (SEE HYMAN, 1923, P. 50); NITRITES ARE GIVEN IN MG. OF NITRITE NITROGEN PER L.; TEMPERATURE 22–23°

"Wholes"	Volume Test Bottle in Cc.	"Eighths"	Volume Test Bottle in Cc.
0.014	122.8	0.010	120.7
0.010	122.5	0.014	120.6

and eighth worms (*E. dorotocephala*), with the results shown in Table VIII.[10] The worms had been starved 11 days. All were washed carefully after cutting and before the nitrite determination. The tests were made in glass-stoppered bottles and extended for the 5 hours immediately following section.

The amount of nitrite produced is sufficient to cause difficulty with Winkler's unmodified method. Since the same amount accumulated with both sets of worms, there need not have been an error in oxygen determinations made on such material if we assume that the same end point would be selected in all determinations with this amount of nitrite present.

The point of greatest interest in Hyman's studies of respiration is their direct support of the axial gradient theory of Child. Specifically

[10] The planarians were supplied and the cuts were made for this and for the following test by Mr. Y. Watanabe, research associate of Dr. C. M. Child. We are indebted to both for their interest and aid.

(1923, pp. 56–67) she showed that with *E. dorotocephala*, the anterior half of the first zoöid consumed about 15 per cent more oxygen in the first 15–24 hours following section than did the posterior half of this zoöid. The anterior half-zoöid extends from a cut made just behind the head to one well in front of the pharynx; the posterior half includes the pharynx region. A piece of the same size as the other two cut from the region of the posterior zoöids was found to consume oxygen at about the same rate as did the anterior piece.

As before, we have not tried to repeat the oxygen-consumption tests. We have determined the amount of nitrite nitrogen produced

TABLE IX

NITRITE NITROGEN, SHOWN AS MG. PER L., PRODUCED BY ANTERIOR AND BY POSTERIOR HALVES OF THE FIRST ZOÖID AND BY PIECES OF THE SAME SIZE CUT FROM THE REGION OF THE POSTERIOR ZOÖIDS; TEMPERATURE 22°

Anterior Half, Produced	First Zoöid; Calculated per Gm.	Posterior, Produced	Half, First Zoöid; Calculated per Gm.	Equal Piece, Produced	Posterior Zoöids; Calculated per Gm.
0.0010	0.00395	0.0015	0.00668	0.0007	0.00394
0.0002	0.00145	0.0018	0.00702	0.0003	0.00141
0.0010	0.00642	0.0020	0.00820	0.0010	0.00671
0.0007*	0.00394*	0.0018*	0.00730*	0.0007*	0.00402*

* Means.

in three lots of worms by pieces from these three regions along the planarian axis. Ten worms were used in each lot. They were approximately 20 mm. long, had been starved for ten days, and were tested in from 116 to 124 cc. of Hull Laboratory well water. The amounts of nitrites produced during the first 24 hours following section are shown in Table IX. Following Hyman's practice, the worms were weighed moist and the amount of nitrite produced is calculated per gram for this 24-hour period. These calculated data do not differ significantly from the crude data.

The amounts of nitrite nitrogen produced even by the middle pieces (posterior half of the first zoöid) is decidedly below the sensitivity of Winkler's unmodified method of nitrites. The smallest amounts of nitrites found approach the limit of sensitivity of the method used for their determination. So far as a direct nitrite error

is concerned, Hyman's data concerning the rate of oxygen consumption of pieces taken at these three points along the axis of *E. dorotocephala* are not open to criticism.

ISOPOD RESPIRATION

Allee (1929) reported on the respiration of recently grouped and recently isolated isopods (*Asellus communis*) in nature. Laboratory tests show that under the conditions of the experiment, isopods can produce 0.11–0.15 mg. per L. of nitrite nitrogen at 21° and from 0.046 to 0.057 mg. per L. at 4.5°. These concentrations would affect the determinations by Winkler's unmodified method, which was the one used, and indicates that the figures given for oxygen consumption for isopods are too low. However, for the purposes for which they were used, the value of the oxygen-consumption data given by Allee, viz., the comparison of the rate of oxygen consumption by recently aggregated isopods with that of the same number of recently isolated individuals, is not affected unless there was differential introduction of nitrites or differential production of this material. The conclusions drawn from the data appear to hold true; these were that the amount of oxygen used by isopods during the first hour after removal from a dense aggregation is decidedly and significantly less than that of the similar lots of isopods during the first hour of their concentration after having been scattered about the bottom of a flowing stream. Such monster aggregations of isopods as Allee worked with are not to be found every year; hence the repetition of this work with the permanganate modification is impossible at present.

RESPIRATION OF OPHIODERMA

In his first work on respiration of *Ophioderma*, Allee (1927) used both the permanganate modification and the unmodified method of Winkler with similar results. In extending these studies to include the breeding season, Allee and Fowler (1932) used the unmodified method only. The differences in results obtained during the two periods of study were interpreted, probably properly, as being due to the changes accompanying the breeding season rather than to methods employed for determining the dissolved oxygen. If a nitrite error had been operating differentially in the work during the breed-

ing season, the grouped animals should have given consistently lower readings of oxygen consumption than did their isolated fellows. With the *Ophioderma* in plain glass dishes, there was a tendency in the opposite direction which was particularly marked at the end of the summer when the oxygen-consumption relations of the grouped and isolated *Ophioderma* again showed practically the same relations revealed in the former study made with the permanganate modification. Direct tests made during the summer of 1934 justified our assumption that the original tests of the two methods which gave similar results regardless of the method used held for the later studies.

RESPIRATION OF DAPHNIA

Fowler (1931), using the unmodified method, found that numbers of *Daphnia* living in a limited amount of $CaCl_2$ solution consume significantly less oxygen than do isolated individuals living in the same volume and concentration of this electrolyte. He further found that isolated individuals living in unaerated $CaCl_2$ solutions in which other *Daphnia* had lived previously have their rate of oxygen consumption lowered to that of groups in the same concentration of raw solution. Aeration of the self-contaminated (homotypically conditioned) salt medium caused isolated *Daphnia* to give a higher rate of oxygen consumption than the accompanying grouped animals. All of these effects are explicable on the assumption of a nitrite error, and on the further assumption that aeration as employed would oxidize the nitrites. That a nitrite error was probably operating is shown by the following data:

One hundred and twenty *Daphnia* that would not pass a 13-mesh screen were placed in 100 cc. of nitrite-free water for 6 hours at a temperature of 20°. Four successive tests made on 2 different days showed a mean amount of 0.0876 ± 0.0117 mg. nitrite nitrogen per L. The oxygen error involved is greater than the difference found by Fowler between the oxygen consumption of grouped and isolated animals. Pending further investigation, Fowler's results, so far as oxygen consumption is concerned, cannot be accepted. Fortunately Fowler's analysis does not depend entirely on his oxygen-consumption data, and his conclusions regarding the rôle of carbon dioxide in protecting *Daphnia* from the toxic effects of $CaCl_2$ remain valid.

RESPIRATION OF FISHES

Schuett (1933), using Winkler's unmodified method, found that with four different species of fishes, when four fishes of the same species were present in approximately 1150 cc. of water, there was less oxygen used per fish than when similar fishes were isolated in the same amount of water. Bowen (1932) obtained the same results, using goldfishes, but found opposite results with the closely schooling

TABLE X

REPETITION OF SCHUETT'S (1933) WORK ON THE SO-CALLED GROUP EFFECT OF CROWDING ON RESPIRATION OF FISHES; IN EACH SET THE RESULTS SHOW THE RATE OF OXYGEN CONSUMPTION PER FISH PER HOUR FIRST ISOLATED AND THEN GROUPED

Set No.	Conditions	Hours of Experiment	Liters Used per Fish	Oxygen Consumed; Cc. per Fish per Hour	Cc. per L.; Lowest O_2 Content at End
I	Isolated	3	1	0.528	3.96
	Grouped	2	1	0.590	1.20
II	Isolated	2	1	0.634	4.52
	Grouped	1	1	0.653	3.58
III	Isolated	1	1	0.618	5.43
	Grouped	2	1	0.590	1.205
IV	Isolated	1	1	0.618	5.43
	Grouped	1	1	0.655	3.46
V	Isolated	1	1	0.584	5.36
	Grouped	1	1	0.653	3.63
VI	Isolated	2	2	0.663	5.10
	Grouped	2	2	0.698	2.96
VII	Isolated	2	2	0.640	5.07
	Grouped	2	2	0.675	3.18

Mean difference = 0.02 ± 0.011 cc. of oxygen per L.; statistical probability = 0.122.

Ameiurus melas. The "group effect" obtained by Schuett vanished when the volume per fish was identical, whether grouped or isolated fishes were studied. This type of "group effect" where the grouped fishes apparently use less oxygen per individual than isolated fishes is precisely according to expectations from the presence of nitrites when the unmodified method of Winkler is used.

This work was accordingly repeated, using the permanganate modification with results as summarized in Table X. In each experimental set the results represent the rate of oxygen consumption per fish per hour for the same set of four goldfishes, first isolated and

then grouped. The water was overlaid with 4 cm. of mineral oil; working temperatures were 23°–25°. The fishes were not fed during the experiments or for 10 days preceding. It is clear that elimination of the nitrite error eliminates the "group effect" found by Schuett and confirmed by Bowen for goldfishes. Schuett has also rechecked[11] these experiments, using the permanganate modification in place of Winkler's unmodified method with substantially the same results as shown in Table X.

As suggested elsewhere (Allee, 1934), the nitrite error, if any, would increase the difference found by Bowen (1932) for aggregated and isolated *Ameiurus melas* since she found a higher rate of oxygen consumption by the grouped animals.

OTHER STUDIES

The use of animals themselves to reduce the oxygen content of water for experimental purposes, which has become a general practice (cf. Helff, 1928; Helff and Stubblefield, 1931; Hiestand, 1931; Amberson, Mayerson, and Scott, 1924), must frequently increase the amount of nitrites present above the point of tolerance for accurate determination of dissolved oxygen by Winkler's unmodified method which was used by these workers. At least we have found that this happens with goldfishes, *Asellus*, *Daphnia*, and crayfishes and, at times, with *Euplanaria dorotocephala*. Whenever these conditions hold, the apparent oxygen consumption as measured by differences between Winkler determinations made at different stages of the process would appear too high in the later determinations. Errors due to accumulating nitrite would affect accurate determination of the end point in any test. Such errors would also cause the point at which oxygen consumption begins to be affected by decreasing oxygen tension to appear to be at a higher concentration than was actually the case. Under certain conditions, respiring animals may be made to appear to be giving off oxygen into the water rather than removing it. In reality they may be increasing the nitrite content, directly or indirectly, and may be removing oxygen. Lacking direct tests, we cannot be sure of the extent, if any, of such errors. The possibility exists and should be guarded against in all future work.

[11] Personal communication.

SUMMARY

1. As a result of a survey of the literature and a restudy of the accuracy of determination of dissolved oxygen in water by Winkler's and by other methods, Winkler's method is found to be sufficiently accurate for use in respiration studies on aquatic organisms, if adequately handled.

2. Standard practice in such studies should require: (*a*) demonstration that there is no iodine absorption or adsorption, or make due allowance for this source of error. (*b*) Water to be analyzed at the end of a respiration test should be demonstrated to have less than 0.005 mg. of nitrite nitrogen per L. or the permanganate modification of Winkler's method should be used. In case of doubt the latter should be standard practice. (*c*) Such waters should also be free of ferrous iron, otherwise the same modification or the bromine modification of Alsterberg should be used.

3. Errors inherent in Winkler's or any other method are more costly with low oxygen content, hence greater care is needed in analyzing such waters.

4. Whenever possible in respiration studies, oxygen determinations should be made by two different methods in order to check for hidden errors. A modification of Van Slyke's method is now available for this purpose (Oesting, 1934).

5. *Euplanaria dorotocephala*, in the concentrations tested, at times gave off sufficient nitrites to affect the determinations of oxygen by Winkler's unmodified method and at times did not. Goldfishes, *Asellus*, *Daphnia*, and crayfishes did so in each test made at room temperatures.

6. With planarians, in the cases which we have tested directly, we have either failed to find nitrites above our revised limit of tolerance, or if present, sharpness of end point aside, they need not have affected Hyman's conclusions regarding oxygen consumption for the given experiments.

7. The studies of Allee and his associates on the effect of numbers present on the rate of respiration also appear to be free from such error except that for that of Schuett (1933), Fowler (1931), and Bowen's (1932) work on the respiration of goldfishes.

8. Lowering oxygen content by allowing animals to respire in a closed vessel may introduce iodine absorption and nitrite errors. The introduction of iron under such conditions should be checked in those cases where there is reason to suspect its presence.

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A MODIFIED VAN SLYKE METHOD FOR THE DETERMINATION OF DISSOLVED OXYGEN AND TOTAL CARBON DIOXIDE IN WATER[1]

(One figure)

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THE need in aquatic respiration studies for determinations of dissolved oxygen and total carbon dioxide in small samples of water led the author to modify the Van Slyke and Neill (1924) manometric procedure for the analysis of the oxygen and carbon dioxide content of the blood to serve this purpose. Van Slyke and Neill (1924) have also pointed out a procedure adapting this apparatus to the analysis of water. The difficulties encountered in the Winkler method from nitrites and dissolved organic matter are well known. The Van Slyke procedure is free from these and has the advantage of requiring only a 5 cc. sample for an oxygen and carbon dioxide determination.

APPARATUS[2]

The apparatus used was of the portable closed manometer type described by Van Slyke (1927). The samples were measured into the reaction chamber with 5 cc. Ostwald-Van Slyke pipettes. A very satisfactory gas-free reagent container, designed and used in the laboratory of Dr. M. E. Hanke, was employed instead of those described by Van Slyke and Neill. Figure 1 will illustrate.

The tip of this apparatus is dipped into a small test tube filled with enough mercury to form a seal.

REAGENTS

Five per cent lactic acid.—Dilute 5 cc. of the C.P. (85 per cent) lactic acid to 100 cc. with distilled water.

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[2] Manufactured by Eimer and Amend, New York.

Gas-free 2N NaOH reagent.—Extract the dissolved gases from the reagent in the Van Slyke apparatus and store in a gas-free reagent container (see Van Slyke and Neill, 1924).

Gas-free hydrosulphite reagent.—Dissolve 10 gm. of sodium hydrosulphite plus 1 gm. of sodium anthraquinone-sulfonate in 50 cc. of 1N KOH in a bottle just large enough, when stoppered, to contain the reagent. Transfer by decantation to the reaction chamber of the Van Slyke apparatus. Extract the dissolved gases and store in a gas-free reagent container. At 30° this reagent will keep for a week; at lower temperatures it will keep for 2 weeks or more, as Sendroy states (1931).

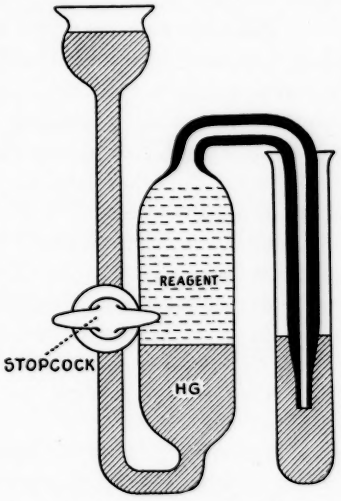

FIG. 1.—Gas-free reagent container

PROCEDURES

In describing the procedures, the stopcock at the top of the reaction chamber will be designated as stopcock *b* and the one controlling the mercury level in the apparatus as stopcock *e*. Van Slyke's articles describe the apparatus and the precautions to be used in its manipulation. This procedure is essentially that described by Van Slyke and Neill (1924). The only modifications are concerned with the concentrations of reagents used which have been altered to suit the special conditions demanded by the analysis of water, but so chosen that the final concentrations are approximately the same as those used by Van Slyke and Neill.

A. FOR THE BLANK

1. Have the reaction chamber filled with mercury, stopcock *b* closed, *e* open, and the leveling bulb at the lower level.

2. By means of graduations on the cup, introduce 6 cc. of distilled water, followed by 1 cc. of 5 per cent lactic acid, into the reaction chamber. Seal stopcock *b* with mercury.

3. Lower the mercury level to the 50 cc. mark by means of the leveling bulb and stopcock *e*, and shake for 3 minutes. Raise the liquid and expel the gas extracted and 0.5 cc. of the acid water mixture. Again seal stopcock *b* with mercury and lower to the 50 cc. mark and shake for 2 minutes.

4. Raise the liquid and expel the gas extracted and 1 cc. of the acid water mixture, leaving 5.5 cc. in the chamber. Reseal stopcock *b* with mercury, lower to the 50 cc. mark, and shake for 2 minutes.

5. Raise the meniscus level of the acid water mixture rapidly at first, and then slowly and evenly to the 0.5 cc. mark, and read p_1 to 0.1 mm. on the manometer. Lower to the 50 cc. mark, shake 1 minute, and repeat the p_1 reading. These should check to 0.5 mm., or the procedure should be repeated until checks are obtained.

6. Lower the liquid level to just below the 2 cc. mark; introduce 1 cc. of the gas-free NaOH into the cup and 0.5 cc. slowly into the chamber. Seal stopcock *b* with mercury and, after 30 seconds for absorption and drainage, raise the meniscus slowly to the 0.5 cc. mark and take the p_2 reading. Repeat this reading by lowering the meniscus below the 2 cc. mark and again setting it at the 0.5 cc. mark. These readings should check to 0.1 mm.

7. After the p_2 reading, record the temperature of the water jacket.

8. Introduce 1 cc. of the hydrosulphite reagent into the cup and 0.5 cc. slowly into the chamber. Seal stopcock *b* with mercury and raise the liquid level to the top of the chamber. Now lower the meniscus below the 2 cc. mark and allow 30 seconds for absorption and drainage. Raise the meniscus slowly to the 0.5 cc. mark and take the p_3 reading. Repeat this reading, as was done with p_2.

9. Wash out the chamber with 10 per cent lactic acid. Rinse with distilled water and it is ready to use again.

B. FOR ANALYSIS OF CO_2 AND O_2 TOGETHER

1. Introduce 2.5 cc. of 5 per cent lactic acid into the cup and 2 cc. into the chamber. Seal stopcock *b* with mercury and lower the mercury level in the chamber to the 50 cc. mark. Shake for 3 minutes. Raise and expel the gas extracted and the liquid into the cup, leaving the capillary filled with mercury.

2. Introduce the 5 cc. sample into the chamber as follows: Allow

the evacuated space at top of the manometer to become completely filled with mercury, and close stopcock *e*. Then place the 5 cc. pipette containing the sample into the cup, obtaining a seal at the bottom by means of a rubber tip fitted to the end of the pipette. Open first the

TABLE I

FACTORS FOR CONVERTING THE PRESSURE READINGS TO CC. OF GAS PER LITER OF WATER, FOR WATER CONTAINING SMALL AMOUNTS OF CARBONATES

Size of sample, 5 cc.
Total volume of solution, 5.5 cc.
Volume of gas, 0.5 cc.

Temp. (Degrees C.)	CO_2	O_2
15	0.1443	0.1248
16	0.1433	0.1244
17	0.1424	0.1239
18	0.1415	0.1235
19	0.1407	0.1230
20	0.1399	0.1226
21	0.1391	0.1222
22	0.1382	0.1217
23	0.1374	0.1213
24	0.1366	0.1208
25	0.1358	0.1204
26	0.1351	0.1200
27	0.1344	0.1195
28	0.1337	0.1191
29	0.1330	0.1187
30	0.1323	0.1183
31	0.1316	0.1179
32	0.1310	0.1175
33	0.1304	0.1171
34	0.1297	0.1168

pipette stopcock and then stopcock *b*, holding the pipette firmly in place. Next, by means of stopcock *e*, control the flow of the sample into the chamber and, when the meniscus is near the low mark of the pipette, close stopcock *e*. The final adjustment of the liquid in the pipette is made by exerting a variable pressure with the pipette, and closing the stopcock of the pipette when the meniscus is exactly at the lower mark. Stopcock *b* is then closed. Now open stopcock *e* and gently raise the pipette 1 or 2 mm. above the bottom of the cup, and,

holding it in this position, allow 0.5 cc. of the 5 per cent lactic acid to flow into the chamber through stopcock *b*, thus washing in the last of the sample around the tip of the pipette. Remove the pipette and seal stopcock *b* with mercury. Lower the mercury level to the 50 c.c mark and shake for 3 minutes.

3. Proceed as directed under steps 5–9, inclusive, of the blank (A)

TABLE II

FACTORS FOR CONVERTING THE PRESSURE READINGS TO CC. OF GAS PER LITER OF WATER FOR WATERS CONTAINING LARGE AMOUNTS OF CARBONATES

Size of sample, 5 cc.
Total volume of solution, 5.5 cc.
Volume of gas, 2.0 cc.

Temp. (Degrees C.)	CO_2	Temp. (Degrees C.)	CO_2
15	0.5661	25	0.5329
16	0.5624	26	0.5300
17	0.5588	27	0.5271
18	0.5552	28	0.5244
19	0.5530	29	0.5217
20	0.5487	30	0.5190
21	0.5456	31	0.5164
22	0.5423	32	0.5140
23	0.5391	33	0.5114
24	0.5360	34	0.5090

C. FOR ANALYSIS OF CO_2 ALONE

Proceed as directed under steps 1 and 2 of B. Then follow steps 5, 6, 7, and 9 of the blank (A). Omit step 8.

D. FOR ANALYSIS OF O_2 ALONE

Proceed as directed under steps 1 and 2 of B, shaking for 4 minutes instead of 3 at the end of step 2. Raise to just below the 2 cc. mark and continue as directed under steps 6, 7, 8, and 9 of the blank (A). Omit step 5.

CALCULATIONS

$p_1 - p_2$(for A) $= C_{CO_2}$. $(p_1 - p_2 - C_{CO_2}) \times \text{Factor}_{co_2} =$ cc. of CO_2 per L.

$p_2 - p_3$(for A) $= C_{O_2}$. $(p_2 - p_3 - C_{O_2}) \times \text{Factor}_{o_2} =$ cc. of O_2 per L.

Factor CO_2 and Factor O_2 are taken from Table I. This table was calculated from the equations and data given in the papers from Van Slyke and his co-workers (1924 and 1927).

Working with sea water and some natural waters, the author found it necessary to measure the carbon dioxide liberated at the 2.0 cc. volume in the reaction chamber instead of the 0.5 cc. volume. Table II gives factors for converting such results to cubic centimeters per liter of carbon dioxide when such a procedure is necessary.

DESCRIPTION OF THE ANALYSES IN TABLE III

The water used in this work was made up from distilled water to contain 50 mg. each of $NaNO_3$, $MgSO_4$, and K_2SO_4 and 100 mg. of $CaCl_2$ per liter. This water tested free from nitrites. Samples 1–8, inclusive, represent duplicate analyses by the Winkler and modified Van Slyke methods for dissolved oxygen. The Winkler method used was that described in the *Official and Tentative Methods of the Association of Official Agricultural Chemists* (1925).

Samples 9–12, inclusive, represent duplicate analyses by the Van Slyke method for carbon dioxide. Two liters of water were taken as sample A, and a liter of this was siphoned into a volumetric flask containing a weighed amount of sodium carbonate as sample B. By the difference in the CO_2 content between A and B, the amount of CO_2 recovered was determined and compared with that added in the form of carbonate.

Samples 13 and 14 represent determinations in which both oxygen and carbon dioxide from the same sample were compared by the methods described above. Winkler determinations were run on both the A and B samples in this case.

SUMMARY

1. A modified Van Slyke method for the analysis of dissolved CO_2 and O_2 is described, and O_2 determinations by this method are compared with those by the Winkler method.

2. A 5 cc. sample is required. An analysis can be completed in $\frac{1}{2}$ hour. The accuracy with CO_2 is 0.1 cc., and with O_2 is 0.06 cc., per liter of water.

TABLE III

SUMMARY OF ANALYSIS*

(Values expressed in cc. per liter)

Sample no.	Winkler method O_2	Modified van Slyke method			
		O_2	CO_2		
			Analysis	Added	Recovered
1	5.93 5.99	5.96 5.93			
2	5.90 5.85	5.88 5.86			
3	4.83 4.80	4.85 4.83			
4	5.60 5.65	5.65 5.66			
5	2.22 2.28	2.26 2.30			
6	3.45 3.53	3.51 3.55			
7	2.46 2.49	2.48 2.49			
8	3.72 3.76	3.74 3.78			
9A			2.79 2.81		
9B			25.73 25.71	22.68	22.92
10A			2.09 2.03		
10B			29.60 29.51	27.99	27.50
11A			1.92 1.95		
11B			25.40 25.43	23.52	23.48
12A			2.19 2.22		

TABLE III—*Continued*

Sample no.	Winkler method O_2	Modified van Slyke method			
		O_2	CO_2		
			Analysis	Added	Recovered
12B			28.34 28.37	26.15	26.31
13A	4.96 4.93	5.15 5.13	1.89 1.85		
13B	4.80 4.81	5.02 5.04	31.17 31.12	29.23	29.26
14A	5.03 5.01	4.98 4.95	2.27 2.23		
14B	5.08 5.08	5.07 5.04	29.30 29.35	27.42	27.08

Oxygen: Mean diff. = 0.038 cc. per liter
Probability = 0.1452

Carbon dioxide: Mean diff. = 0.073 cc. per liter†
Probability = 0.4558

* In each case the B sample represents 1 liter of the A sample, to which has been added a weighed portion of Na_2CO_3.

† A probability of greater than 0.05 has no statistical significance.

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RESPIRATION AND DEVELOPMENT OF INDIVIDUAL MUD-DAUBER WASP LARVAE FOLLOWING X-IRRADIATION[1]

(One figure)

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IN AN earlier report (Bodine and Evans, 1932) certain correlations between respiratory rates and visible changes during development of the mud-dauber wasp larva (*Sceliphron caementarium*) were pointed out. The purpose of this paper is to report the effects on the respiration and visible development of the larvae following different doses of X-irradiation.

The larvae (approximately one hundred, all in the same stage of development) were collected in the fall of the year and were divided into two groups. Those of the first group were placed at a constant temperature of 28° C., and designated as the "diapause lot" since they usually remained inactive for several weeks before beginning development. The larvae of the second group were left at a temperature of 8° C. until all the diapause larvae of group 1 had developed. By this time these group 2 larvae would begin development at once when placed and left at 28° C. (Bodine and Evans, 1932).

The respiratory rates of individual larvae were determined daily by means of the modified Krogh differential manometer at 28° C. The same manometer was used throughout for both irradiated and control larvae. For further details of methods, etc., the reader is referred to previous article (Bodine and Evans, 1932). The external anatomy of each larva was carefully studied and recorded and in this fashion correlations could be made between development and physiological response.

The radiations employed were neither extremely hard nor extremely soft. A. Coolidge, tungsten-target, broad-focus tube was

[1] This investigation has been aided by a grant from the Committee on Radiations of the National Research Council.

activated at 130 kv. and 5 ma. The organisms were contained in gelatin capsules and were supported on a cloth 35 cm. beneath the target of the tube and some 2 feet above the floor. No filter was used in the preliminary experiments. The intensity was usually 165 *r*-units

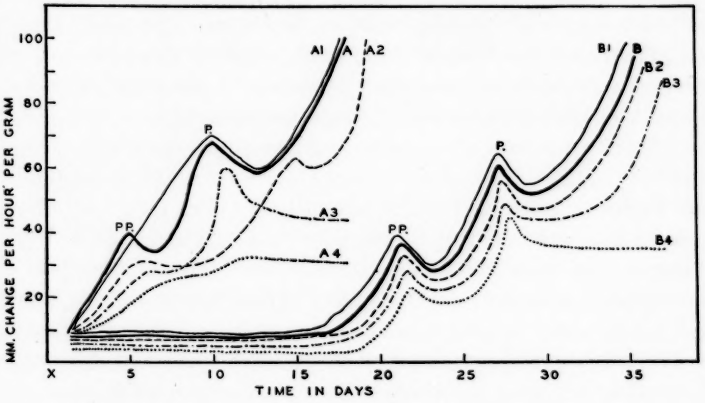

FIG. 1.—A composite figure, based upon results for individual larva, showing typical effects of X-irradiation on the rates of oxygen consumption and growth of developing (non-diapause) and blocked (diapause) mud-dauber wasp larvae. Ordinates, mm. change manometer fluid per hour per gram. Abscissae, time in days indicated. *X* on abscissae indicates time of irradiation. Letters *PP* designate pre-pupal stage. *P* designates pupal stage. Curves marked with letter *A* refer to developing (non-diapause) larvae. Those marked with letter *B* refer to blocked (diapause) larvae. A = control, non-irradiated, developing larva. A_1 = same, given 300 *r*-units. A_2 = same, given 600–700 *r*-units. A_3 = same, given 800–1,000 *r*-units. A_4 = same, given 1,200 *r*-units. B = control, non-irradiated diapause larva. B_1 = same, given 300 *r*-units. B_2 = same, given 600–700 *r*-units. B_3 = same, given 800–1,000 *r*-units. B_4 = same, given 1,200 *r*-units. For further description see text.

per minute, and was measured by the commercial "dosimeter." The dosage was increased by lengthening the exposure time.

RESULTS

Rather marked individual variations in response of the two types of larvae to X-irradiation occur, but in general the trends are always in the same direction. Typical results will, therefore, be presented rather than detailed individual cases (Fig. 1).

NON-DIAPAUSE OR DEVELOPING LARVAE (GROUP 2)

The non-diapause larvae in the present experiments differ from the diapause ones by virtue of the fact that development begins immediately after irradiation. In Figure 1 are graphically shown the effects of various doses of X-ray on the oxygen consumption as well as upon the visible development of the larvae. The point marked *PP* designates the time at which the pre-pupal stage is reached, while the point *P* indicates the formation of the pupa. It will be noted that the characteristic U-shaped oxygen-consumption curves for these two points are obtained as previously indicated (Bodine and Evans, 1932). Very light dosages (50–200 *r*-units) failed to alter the visible development or the respiratory cycles from that shown by non-irradiated control larvae (Fig. 1, *A*). When slightly higher dosages (250–400 *r*-units) were administered, some evidences of acceleration were indicated (Fig. 1, *A*1). The effect on the respiratory cycle is shown by the absence of the drop or U-shape which ordinarily is shown for oxygen consumption at the pre-pupal stage. The pupal cycle, however, was not altered. Visible development was not consistently speeded up although a few individuals did exhibit both developmental as well as respiratory acceleration. Slightly higher intensities of irradiation (400–500 *r*-units) failed to alter noticeably the visible development or the respiratory cycles, while still higher dosages (1,200–1,500 *r*-units) were all markedly injurious.

In curve *A*2 are graphically shown typical effects resulting from irradiations of 600–700 *r*-units. Although the time of metamorphosis was slightly delayed in such cases, the pupae usually emerged visibly complete.

When the dosage was increased to 800–1,000 *r*-units, as shown graphically in curve *A*3, the pre-pupa cycle proceeded at approximately the normal rate but the pupae failed to emerge. There was apparently no recovery with time as the pupae remained permanently in the abortive condition. When still higher dosages (1,200–1,500 *r*-units) were administered, the irreversible injury was apparent in the early pre-pupal cycle, as shown graphically in curve *A*4.

DIAPAUSE LARVAE (GROUP 1)

Since the length of the diapause period varies, the latent period, or period between irradiation and active development of the irradiated

larvae, seems unimportant at this time. Initiation of development after irradiation falls within the time range of the non-irradiated controls. The effects of irradiation on diapause larvae are shown graphically in Figure 1. It will be seen from an examination of this figure that more of the curves for irradiated larvae are similar to those for the controls or non-irradiated forms than in the case of the non-diapause type. Dosages of 1,200–1,300 *r*-units injure the larva irreversibly, the effects being most evident at the time of metamorphosis (Fig. 1, B_4). It will be recalled that similar effects were produced in the non-diapause larvae (Fig. 1, A_3) by only 800–1,000 *r*-units of irradiation. Dosages of 800–1,000 *r*-units affect the diapause larva little if any (Fig. 1, B_3; cf. A_3). Below 700 *r*-units the effects on the diapause larva can scarcely be shown to exist (B_3, B_2, B_1).

It is quite obvious that the diapause larvae are not as radiosensitive as the non-diapause ones. The two types were morphologically similar at the time of irradiation and their respiratory rates were also extremely comparable, but the subsequent responses to irradiation vary to an extreme degree. A period of developmental inactivity following irradiation in the diapause organism is perhaps the greatest apparent difference between the two types of larvae.

Several workers have recently found that recovery takes place if irradiated material is kept inactive for a time following irradiation (Strangeways and Fell, 1927; Packard, 1930; Henshaw, 1932; and Evans, 1934). The recent work of Henshaw and Francis (1933) indicates that lessened metabolism and cell division preceding and during the irradiation do not noticeably decrease radiosensitivity, if the tissues are allowed to develop immediately following the irradiation.

The diapause larvae in the present experiments differed from the non-diapause organisms in that they were inherently different at the time of the irradiation and also in that development was delayed until some time had elapsed after the treatment. In order to determine which of these two factors was the more important, the following experiments were tried. Non-diapause larvae were kept inactive for a time following irradiation by placing them back at low temperature (8° C.) while similar groups were allowed to begin development immediately after irradiation. The physiological conditions

of the two types of organisms were thus relatively the same previous to and during the irradiation, the only difference being that immediately following the irradiation one group was kept inactive for a time, while the other was allowed to start development at once. It was invariably found that recovery did take place in the organisms kept inactive following the irradiation. The accelerative effect of the light dosages disappeared after only three or four days of inactivity. The retarding effect of 600–700 *r*-units disappeared after a week or more of inactivity. Even the destructive effect of 1,000 *r*-units was absent after a month's inactivity following the irradiation. That there is a definite range within which recovery is possible is shown by the fact that the harmful effect of 2,000 *r*-units failed to disappear after even several months of inactivity at low temperature (8° C.).

These results agree very closely with those found for developing grasshopper eggs (Evans, 1934). In both the mud-dauber wasp and the grasshopper experiments it was found that light dosages decreased in effectiveness with time even though the organisms were developing. It was also found in both cases that heavy dosages caused injuries which seemed to increase with time if the organisms were developing. Even extremely heavy dosages, within limits, tended to decrease with time if the organism was inactive.

CONCLUSIONS

1. Larvae of the mud-dauber wasp, *Sceliphron caementarium*, in the actively developing and diapause or blocked conditions have been subjected to different dosages of X-rays and their rates of oxygen consumption and growth studied.

2. Actively developing larvae are more sensitive to X-irradiation than blocked or "diapause" larvae.

3. Organisms, irradiated and immediately subjected to periods of normal or artificially produced developmental blocks, show marked powers of resistance to, or recovery from, the harmful effects of X-irradiation normally shown by developing organisms.

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RESPIRATION AND VISIBLE DEVELOPMENT OF INDIVIDUAL GRASSHOPPER EGGS FOLLOWING X-IRRADIATION[1]

(One plate; seven figures)

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AN IMPORTANT step in the investigation of biological effects of X-rays is to determine whether the materials and methods employed will yield results which will correlate the extent of the effect with the amount of the irradiation. Many investigators have selected one particular biological response and have measured the variation of this character at a certain time or stage in the experiment. The percentage of viability at a particular time after irradiation has been used as one end-point, and the results have been fairly constant. For example, Packard (1926) and Failla and Henshaw (1931) have used the percentage of hatching of *Drosophila* eggs. Koidsumi (1930) studied the killing-rate on developing and adult *Dacus* flies. This method, as stated by Packard (1931), gives results which indicate that effects of X-rays on the viability of organisms are subject to the same variation as are biological responses to other external agencies. Henshaw (1932*a*) has recently shown that developing *Drosophila* embryos were especially radiosensitive at gastrulation. Therefore, unless the material is homogeneous as to the developmental condition of the individuals, this biological variation may be exaggerated.

Other investigations have been concerned with a particular function of the test organism, and the results indicated that the effect was proportional to the intensity of the irradiation. Cattell (1931) has reported that growth of different parts of wheat seedlings was proportionately retarded by increasing amounts of irradiation. Hen-

[1] This investigation has been aided by a grant of the Committee on Radiations of the National Research Council.

[2] The writer wishes to express his gratitude to Professor J. H. Bodine, who suggested this type of investigation and who has helped in many ways to make this report possible.

shaw (1932*b*) found that the time of the first cleavage of the *Arbacia* egg was proportionately delayed by increasing the intensity of the irradiation. Obreshkove and King (1932) concluded that growth and gaseous metabolism of a certain cladoceran was proportionately retarded by increasing amounts of irradiation.

The above-mentioned papers are by no means all of those that have been written concerning this topic, but are representative of the various methods of studying quantitatively the biological effects of X-rays. One is struck with the fact that only end-points have been extensively studied (see Packard, 1931). It is obviously desirable that the progression of the effects be followed. An approach to such a method has been briefly described by Blumenthal and Williams (1928). The average respiration and developmental rates were followed in grasshopper eggs from the time of irradiation until the controls began to hatch. Another type of investigation is that of Hussey, Thompson, Tennant, and Campbell (1927, 1932), who found a maximum intensity of irradiation for the lengthening of the prepupal period of *Drosophila*. The finding that, above a certain intensity of irradiation, the effect was not proportionately increased may indicate that this particular criterion had a limited capacity. It is, therefore, advisable to follow the irradiated organisms through several physiological cycles and to use more than one criterion of effect.

Since the time of the first report on irradiation of grasshopper eggs (Blumenthal and Williams, 1928), much has been learned concerning the details of the development of this particular insect (see Bodine, 1929, 1932*a*, 1932*b;* Slifer, 1932*a*). Burkholder (1934) has found that the rate of respiration and development may vary for individuals at a particular stage and that later cycles of development may compensate for the variations of the previous ones. It is, therefore, important that individual eggs be followed from the time of the irradiation until the culmination of the effects. It is also necessary that the development up to the time of the experiment be controlled as much as possible. If the developing eggs have a common environmental history the later stages will tend to be more uniform than they would otherwise.

The object of this investigation was to study the effect of increas-

ing amounts of X-ray irradiation on the subsequent visible development and respiration of individual grasshopper eggs. The eggs were treated at a stage of development when the subsequent changes could be easily followed. Effects were noted on the rate of oxygen consumption, movement, growth, and the ability to hatch. An effort was made to determine whether the time of appearance, the extent, or the duration of the effects change with increasing amounts of irradiation.

MATERIALS AND METHODS

The material employed in these experiments was the egg of the common grasshopper, *Melanoplus differentialis*. This organism has been studied quite extensively by Dr. Bodine and his associates and is excellent material for certain physiological experiments. It has been found, by the various workers in this field, that most of the eggs of each pod suffer an interruption in their development (diapause) after a certain stage has been reached. This diapause (see Bodine, 1932*b*) asserts itself at the time when the embryo would otherwise begin blastokinesis. The duration of the diapause varies among individuals from the same pod. It has been known for some time that such a diapause is not evident after long exposures to subdevelopmental temperatures (below 10° C.). The eggs used in these experiments were allowed to develop for more than 20 days at 28° C. By this time the diapause embryos were definitely inactive and the non-diapause ones had started blastokinesis. The diapause eggs were placed in a low-temperature (10° C.) chamber. At this temperature no visible development occurred. After several months in the low temperature the embryos began development uniformly when returned to 28° C.

The visible changes in the eggs were observed through a binocular dissecting microscope. The reader is referred to Wheeler (1893) and Slifer (1932*b*) for the details of these morphological changes. Three stages of this development are especially distinct. These stages have been designated as (1) "blastokinesis," (2) "yolk engulfment," and (3) "externally complete." Blastokinesis usually consisted of two movements—revolution and rotation. Diagrammatic sketches were made of the visible changes in normally developing embryos. These sketches are shown in Plate I. It will be noticed that at the up-

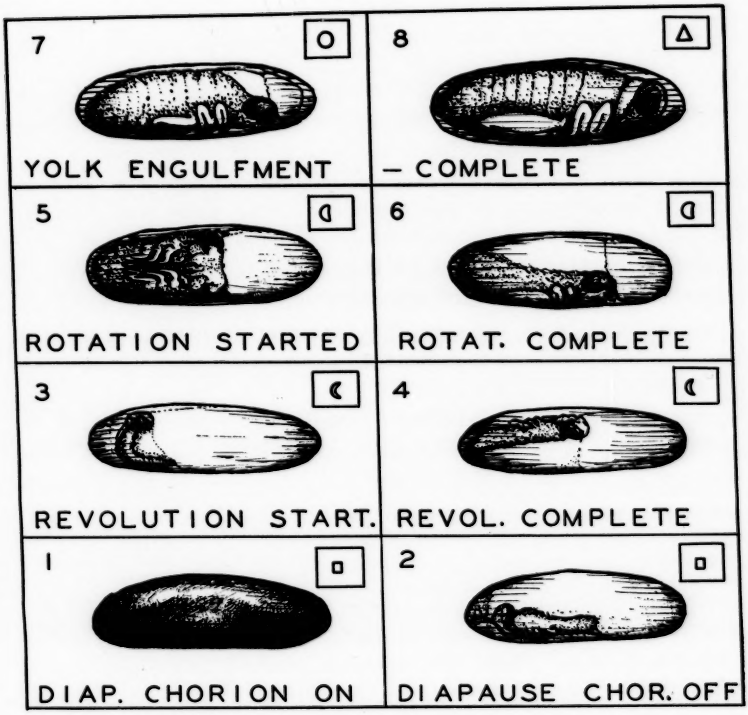
per right of each sketch a symbol has been drawn. These symbols are used in the following graphs to indicate the visible condition of the embryo at the time of the determination of its oxygen consumption rate. For example, consider the control in Figure 1. The character and position of the symbol on the second day indicate that the embryo was apparently inactive and was using oxygen at the rate of 5 mm. per hour. On the sixth day the character and position indicate that the embryo was engulfing yolk and was using oxygen at the rate of 9 mm. change per hour. It is possible to follow, by this means, the changes in visible development on the same graph with the changes in the respiratory rate.

The oxygen consumption of groups of eggs was determined by means of the modified Krogh manometer (Bodine and Orr, 1925). Rates of single eggs were determined by means of a smaller manometer built on the same principle. These micromanometers were so sensitive that a pressure change was evident after only an hour with a single egg. Determinations were made at the same temperature at which the eggs were kept during their entire developmental period (28° C.). A determination usually lasted about 5 hours. The number of millimeters change in the position of the manometer fluid divided by the number of hours of the determination was taken as an indication of the rate of oxygen consumption of the egg for that period. All determinations of an individual egg were made with the same manometer. When the eggs were not in the manometers, they were kept on moist cotton in individual test tubes which were partially submerged in the same water bath in which the determinations were made. The rate of oxygen consumption of each egg usually increased rapidly the first day at 28° C. after it had been taken from the low temperature (9°–10° C.). Toward the end of the second day the rate was still higher and some movement of the lateral surfaces of the embryo was visible. Usually on the third day the embryo revolved and rotated. During this time the oxygen consumption rate was rather high but remained fairly constant until about the end of the first week, by which time the embryo had completely engulfed the yolk. The oxygen consumption rate was accelerated on or about the fifteenth day, and the embryo usually hatched within the next few days. These changes are indicated in the control curves of

PLATE I

Diagrammatic sketches of visible condition of embryo at different stages of development studied. No. 1 is of an egg just removed from the low temperature at the beginning of the experiment. The micropyle is at the left, and the egg is resting on its concave surface. This orientation is continued in the following sketches. The eyes of the embryo can be seen dimly through the chorion. By removing the chorion (Slifer, 1932*a*), the whole embryo is clearly visible through the chitinous cuticle, and one can follow the growth and movements in detail. The embryo lies beneath the yolk on the concave surface of the egg. The head of the embryo is toward the micropyle. Nos. 3 and 4 picture the movement of the embryo around the yolk. Embryo lies above the yolk now, at the micropyle end. Embryo has enlarged. Movements continue, and embryo rotates (Nos. 5 and 6) until it comes to lie once more on the concave surface of the egg. Embryo now begins to grow rapidly in anterior and lateral directions. Movements continue until all of the yolk is engulfed within the embryo (Nos. 7 and 8).

PLATE I


7
YOLK ENGULFMENT
8
– COMPLETE
5
ROTATION STARTED
6
ROTAT. COMPLETE
3
REVOLUTION START.
4
REVOL. COMPLETE
1
DIAP. CHORION ON
2
DIAPAUSE CHOR. OFF

Figures 1–7. It appeared that, in general, accelerations of oxygen consumption rates were a little in advance of the visible morphology in representing developmental changes.

The eggs were irradiated on the day that they were removed from the low temperature. The respiration of the controls at this time indicated that they had recovered from the effects of the low temperature, but there was no visible indication of activity having started. The organisms were supported, 35 cm. beneath the target of the X-ray tube, on a cloth fastened 3 feet above the floor. This arrangement was devised to avoid as much back-scatter as possible. The X-ray machine employed was a Snook unit with mechanical rectification. A broad-focus, tungsten-target Coolidge tube was activated at 130 kv. and 5 ma. Some of the experiments were repeated, using a similar machine at the State University of Iowa Hospital; and the same results were obtained with both machines.[3] The X-radiation was unfiltered, with the exception of 2 mm. of hard-pressed cardboard, which was used to protect the irradiated material from excessive heating. The intensity of the X-ray output was determined by means of the commercial Fricke-Glasser "dosimeter" and the "*r*-meter." The intensity was usually 165 *r*-units per minute. The amount of irradiation was increased by lengthening the exposure time.

EXPERIMENTAL RESULTS

Several groups of three to twenty eggs each were irradiated with amounts ranging from 165 to 6,000 *r*-units. The effects on respiration and visible development following the irradiations were noted. This was done to obtain results which would indicate the general effects of the various amounts of irradiation. Another purpose of these preliminary experiments was to determine appropriate amounts to be employed in the experiments on individual eggs. After the general response to various amounts of irradiation had been determined, it was decided to follow individuals after irradiations of—less than 300, 300, between 300 and 1,000, 1,000, 2,000, 3,000, 4,000, 5,000, and 6,000 *r*-units. One manometer was employed for each

[3] The writer wishes to express his sincere thanks to Dr. H. Dabney Kerr and his associates, of the Department of Roentgenology, State University of Iowa Hospital, for their assistance in this phase of the investigation.

irradiated egg and its control. Each egg was followed for at least 25 days after irradiation. The time and equipment necessary for each experiment limited the data to six to ten individual experiments for each amount of irradiation employed. There was some variation among individuals that were irradiated with small amounts, but the responses to the heavier doses were extremely uniform. Eggs irradiated with less than 1,000 *r*-units usually hatched, and such amounts of irradiation will be considered here as non-lethal doses. Amounts of more than 1,000 *r*-units prohibited hatching, and these will be considered as lethal doses.

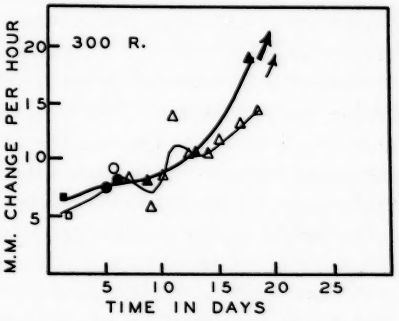


FIG. 1.—Curves showing typical result of irradiating an egg with 300 *r*-units of X-rays. Solid line and solid symbols refer to irradiated egg. Light line and open symbols refer to control egg. Position of symbol indicates rate of oxygen consumed at that time. Character of symbol indicates morphological stage as shown in Plate I. Arrow indicates hatching. No evidence of inhibition of respiratory rate, development, or ability to hatch.

Effects of amounts of irradiation of less than 2,000 r-units.—A typical experiment in which the respiration and visible developmental changes were followed from the time of irradiation until hatching is shown in Figure 1. The trend of the respiratory rate during development is indicated by the slope of the line. The heavy line indicates the respiratory curve for the irradiated egg, and the lighter line is that of the control. The actual determinations of the respiratory rate is indicated by the position of the symbols on the ordinates. The solid symbols refer to the irradiated egg, and the open symbols refer to the control. It is seen that the character of symbols change with time (indicated by the abscissae). The character of the symbols refer to the visible conditions of the embryo, as indicated in the upper right of each drawing in Plate I. Figure 1 is interpreted as indicating that the irradiation of 300 *r*-units did not noticeably affect the

respiration or the visible developmental changes. The other five cases were very similar to those described above.

It is possible that the above-mentioned result is, in reality, an indication of a neutralization of accelerating and retarding tendencies. Five of six individual eggs indicated increased respiration for the first few days following irradiations of less than 300 *r*-units. The visible changes were not noticeably speeded up, and the later respiration was similar to that of the controls. The same number of eggs were studied following irradiations of more than 300 and less than 1,000 *r*-units. The results indicated that respiration and visible development were retarded in these cases for the first 5 or 6 days following the irradiation. The effect disappeared in time as development continued, and the last few says of development were much the same in irradiated eggs as in the controls. The extent of the retarding effect is similar to that shown in the first 15 days of Figure 2.

One might expect that as the amount of irradiation was increased to 1,000 *r*-units the effect would be to further retard the appearance of the latter stages of development. Such was not the case. Intensities of 1,000 *r*-units did not further retard blastokinesis. The additional effect was evident only later when the embryo failed to hatch. This is shown in Figure 2. The rates of respiration and of visible development were similar to that of the controls during the second week of development; but at the time the controls were beginning to hatch, the oxygen consumption rates of the irradiated embryos dropped, and all visible movements ceased within a few days.

Several investigators (Canti and Spear, 1929; Packard, 1931; and Butler, 1932) have found results which apparently agree with the foregoing findings. These investigators found that further mitosis of active tissue was immediately suppressed by irradiation and that after a time the mitotic activity was resumed.

It has been indicated in several reports that insect larvae, after irradiation, lived until the time of pupation, or of ecdysis, and then suddenly died (Mavor, 1927); Packard, 1931; Koidsumi, 1930). These investigators seem to agree that, in insect development at least, it is possible for lethal effects to be delayed until times of great physiological disturbances such as metamorphosis.

The effects of the non-lethal doses of irradiation on the grass-

hopper eggs will be regarded as having temporarily altered processes immediately following the irradiation. The additional effects of the lowest lethal dose were not evident until the time of hatching of the controls.

Results of irradiating eggs with 2,000–6,000 r-units.—The effects of irradiations of 2,000 *r*-units (Fig. 3) indicate that the permanent suppression was noticeable a few days earlier than was the case with the lower lethal dose (1,000 *r*-units). The immediate retardation in visible development and respiration was not noticeably increased by the 2,000 *r*-units irradiation. The greater intensity of the effect was apparent during the last 4 days of the development. The rates of respiration were noticeably retarded even before the time of the hatching of the controls.

The amount of irradiation was increased by another 1,000 *r*-units (total, 3,000 *r*-units), and the results are shown in Figure 4. It is seen that the irradiated embryo was able to complete blastokinesis and the engulfment of the yolk. The embryo was externally complete (as indicated by the triangles). The rate of oxygen consumption, however, failed to increase as in the controls when this visible condition was reached. In several cases the internal structure of the embryo at the time of death was visibly incomplete. Two of the embryos still had a small plug of yolk protruding above the head when further growth was apparently blocked.

The results of irradiation of 4,000 *r*-units indicate clearly that the effect of additional amounts of irradiation was associated with a more rapid appearance of the permanent suppression on the respiration and visible development. This may be seen by comparing Figure 5 with Figures 3 and 4. After the irradiation of 4,000 *r*-units the embryos were still visibly incomplete when they stopped developing. This is shown in Figure 5 in that the final condition of the irradiated embryo (represented by the circle) was still of the yolk engulfment stage. The control had reached the complete stage (represented by the triangles) some 10 days before. The yolk was only partially engulfed in all of the irradiated eggs, and the appendages were not of full size.

The permanent suppression was noted at a still earlier time following irradiations of 5,000 *r*-units. A typical case is shown in

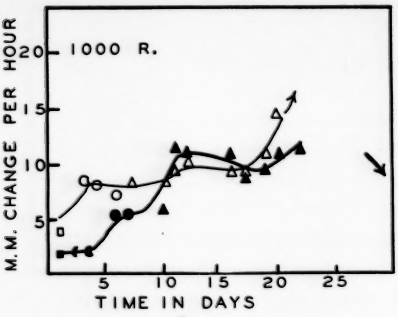


FIG. 2

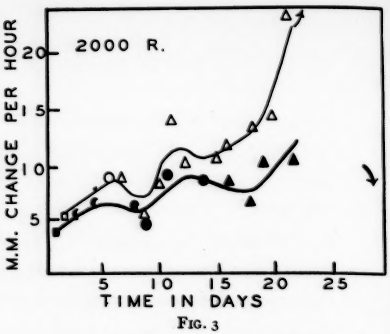


FIG. 3

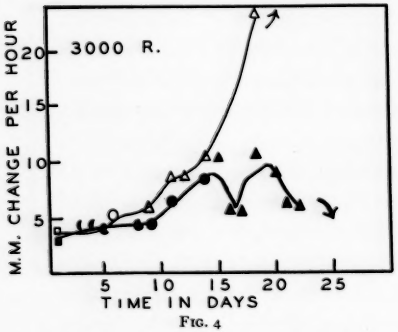


FIG. 4

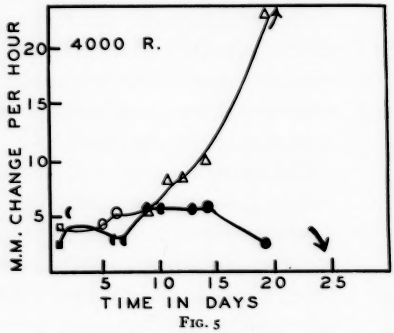


FIG. 5

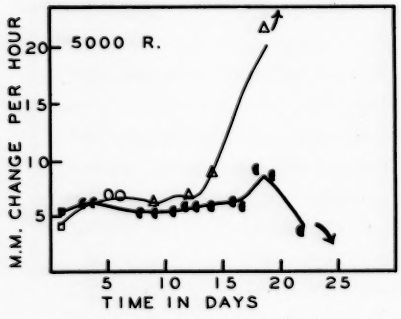


FIG. 6

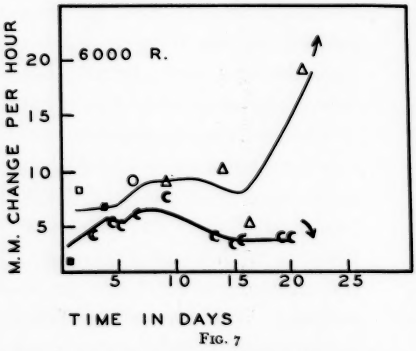


FIG. 7

FIG. 2.—Curves showing typical effect of irradiating an egg with 1,000 *r*-units. Downward arrow indicates cessation of movement, or death. Other symbols same as in Figure 1. Indicates immediate inhibition of respiration and development with later recovery. Also indicates failure of embryo to hatch.

FIG. 3.—Curves showing typical effects of 2,000 *r*-units. Symbols as in Figure 2. Respiratory rate effected in complete stage. Embryo failed to hatch.

FIG. 4.—Curves showing typical effects of irradiating an egg with 3,000 *r*-units of X-rays. Explanation of figures in Figure 1. Ultimate suppression of respiration, development, and hatching is indicated.

FIG. 5.—Curves showing typical effects of an irradiation of 4,000 *r*-units. Embryo died in incomplete condition, in that yolk was not entirely engulfed. Respiratory rate remained low after tenth day.

FIG. 6.—Curves showing typical effects on an irradiation of 5,000 *r*-units. Respiration and development suppressed after rotation—fifth day.

FIG. 7.—Curves showing typical effects of an irradiation of 6,000 *r*-units. Suppression of respiration and development after the stage of revolution.

Figure 6. Almost all growth was suppressed from the beginning of the experiment. All of the embryos were small and the organs remained rudimentary. All embryos, however, went through the two movements of blastokinesis at the same time as the controls.

After an irradiation of 6,000 *r*-units, all visible growth was completely suppressed and death usually occurred before the embryo had completed blastokinesis. Like the case shown in Figure 7, most of the embryos revolved around the yolk but failed to rotate or to enlarge.

The immediate movements of blastokinesis were evidently very resistant to irradiation in these experiments. Eggs irradiated with even 10,000 *r*-units gave indication of starting to revolve. None of these, however, were able to move more than one-third of the way around the yolk.

It will be recalled that all of the irradiations employed above were ultimately lethal. It should also be kept in mind that the first indication of this lethal effect was a drop in the respiratory rate occurring just a few days before the controls hatched. It may be said that the lethal effect was apparent only after a certain latent period. It appears that this latent period was shortened by increasing the amount of irradiation. There are evidences from other sources which indicate that this response to irradiation may be general. Prime (1916) found that the lethal effect on embryo chick heart *in vitro* appeared quicker after heavier quantities or irradiation had been applied. It also appears from the data of Koidsumi (1930) that the heavier doses caused the organisms to die at an earlier stage of development. It is possible in experiments, as those above, to use the time of appearance of permanent suppression following the irradiation as an indication of the intensity of the effect of irradiations.

The conditions of the experiments reported here were arbitrarily selected. It is of interest to know in what ways these conditions have effected the results obtained. It is well to know whether the results obtained were directly or indirectly related to X radiation. The criteria used were the respiratory rates and visible developmental rates following the irradiation to the time of hatching of the controls. The question now arises as to whether different results would

have been obtained had some factor following irradiation been different. The first factor to be changed was that of the temperature following irradiations. In a few preliminary experiments eggs were returned to the low temperature (9°–10° C.) immediately following the irradiation. Developmental activity was suppressed in these eggs until they were subjected to the developmental temperature employed (28° C.). It will be recalled that in the preceding experiments when development was allowed to start immediately following the irradiation, a dose of 2,000 *r*-units was definitely lethal. Eggs which had been kept inactive (by the low temperature) for a few weeks following irradiations of 2,000 *r*-units developed at the later date much like the controls. Eggs irradiated with 5,000 and some with 6,000 *r*-units were treated as above. These eggs did not hatch, but they did develop further than those which had been allowed to start immediately after the irradiation. It appears from these preliminary experiments that some recovery may take place if the eggs are kept inactive for a time following the irradiation. It has been demonstrated by Henshaw (1932*b*) that one effect of irradiation, suppression of first cleavage of *Arbacia* eggs, was decreased by delaying the activation of the organisms. Packard (1930) has reported that in *Paramecia* the lethal effect is apparent sooner in the more rapidly growing cultures. These experiments indicate that the degree of mitotic activity following irradiation may be an important factor in radiosensitivity. It was noted in the present investigation that recovery was quicker and more complete in those eggs irradiated with light amounts than in those treated with the heavier doses.

The question now arises as to whether recovery could take place at any time after irradiation by making the embryo inactive for a time. Eggs irradiated with 5,000 *r*-units and others treated with 6,000 *r*-units were allowed to develop for 5 days and then placed back at the low temperature for a month. When the irradiated eggs were placed again at the developmental temperature, they gave no noticeable effects of recovery. These experiments indicate that recovery took place when the organisms were kept inactive for a time

immediately following the irradiation. They also indicate that after the injured embryo had been allowed to develop for several days it could no longer recover by being made inactive for a time. Henshaw and Francis (1933) found that applying low temperatures to wheat seedlings, before and during irradiation, did not make them more resistant than seedlings which were growing at the time of irradiation. It is apparent that in both cases the seedlings were allowed to grow immediately following the treatment. In the case of the grasshopper eggs it appears that radiosensitivity, as measured by later development, was not so much a matter of the developmental condition of the embryo at the time of the irradiation as it was of the activity of the organism immediately following the irradiation.

CONCLUSIONS

1. Both the rate of visible development and the rate of oxygen consumption of the individual eggs following irradiations showed changes which were apparently associated with the amount of irradiation employed.

2. Light doses of irradiation affected the immediate respiration and developmental rate, but the effects lasted only a few days.

3. The first permanent effects noted, as the amount of irradiation was increased, were the suppression of further respiration and visible development at the time when hatching would normally occur. This cessation of growth was not apparent until the time of hatching, and may be thought of as a delayed lethal effect.

4. The amount of irradiation was increased above that which was ultimately lethal, and it was found that the latent period was correspondingly shortened. The heaviest doses suppressed all visible growth, but the first movements of blastokinesis were found to be extremely resistant.

5. Evidence is presented which indicates that the particular quantitative relationships of the effects to the amounts of irradiation were due to the fact that the organisms were allowed to begin development immediately following the treatment.

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CERTAIN PHYSIOLOGICAL EFFECTS OF X-RAYS ON EUPLANARIA DOROTOCEPHALA. I. DIFFERENCES IN SUSCEPTIBILITY[1]

(Three figures)

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THE present paper concerns a series of experiments in which the flatworm *Euplanaria dorotocephala* was exposed to various dosages of X-rays. The object of the experiments was primarily to determine whether these animals show a differential susceptibility to X-rays as they do to many other agents.

MATERIALS AND METHODS

E. dorotocephala is a triclad turbellarian found in springs or among the vegetation of shallow spring-fed streams. It has been extensively experimented upon, especially by Child and his students, but generally under the name *Planaria dorotocephala*. The placing of it in the genus *Euplanaria* is in conformity with the classification of Hyman (1931).

The animals used were taken in the fall of the year from a spring near Rockford, Illinois. In the laboratory they were kept in large pans of well water and were fed liver three times a week. After each feeding the pans containing the worms were thoroughly rinsed and fresh well water was added. Care also was taken to keep the water cool at all times. When an experiment was to be run, a stock pan was set aside and the animals in it were no longer fed. Preliminary experiments had shown that food in the gastrovascular cavity of the animals at the time of the irradiation introduced undesirable complications into the experiments. Furthermore, it seemed probable that more uniform physiological conditions would prevail after a starvation period of two weeks than would be the case after a similar

[1] The writer is deeply indebted to Professor C. M. Child for invaluable suggestions and criticisms and for his stimulating interest in the investigation. The writer also wishes to thank the Department of Medicine of the University of Chicago for the use of one of their X-ray machines.

period of feeding. In fact, experiments of Child and others have shown that to be the case.

On the fourteenth day of starvation, one hundred and twenty animals, measuring between 18 and 20 mm. in length when crawling on a surface film of water, were chosen from the starvation stock. Without further selection they were divided into lots of twenty animals each. Of the six lots obtained, one, Lot I, was kept as a control while the remaining five, Lots II–VI, were exposed to 2, 4, 6, 8, and 10 minutes of raying, respectively. While irradiated the animals were crawling on a moist glass surface. Since twenty were exposed at one time, one occasionally crawled over another, shielding it temporarily from the rays, but the protection thus effected did not seem sufficient to warrant the raying of them one at a time.

The X-ray machine used was operated during each irradiation at 85 peak kv. (alternating potential) and at 5 ma., and the animals irradiated were exposed in every case without filter at a distance of 35 cm. from the target. Thus the physical "set-up" was kept constant and, as already indicated, only the time of the exposure was varied. The tube under the conditions mentioned delivered approximately 86.4 Roentgen units per minute.

Following the irradiations, the twenty control animals and the hundred exposed worms were placed individually in Syracuse watch glasses. Enough water was added to each dish to cover the animal in it, and the starvation which had been initiated two weeks prior to an irradiation was continued. Every other day each animal was examined under a binocular dissection microscope and the effects of the rays, if any, were studied and recorded. Since the effects that appeared revealed themselves very slowly, the observations on a given animal extended in most cases over a period of about two months.

RESULTS

Although the data of the tables that follow are presented as the results of a single set of experiments, they are actually the combined data of two identical sets run approximately three months apart. Thus each experimental lot is represented by forty animals instead of twenty. The bringing-together of the data seemed justified on the basis of the close agreement of the results of the second set with those of the first.

Table I gives the percentage of animals that showed an effect of the rays. In Figure 1 they are plotted against exposure time. It will be seen that there is approximately a 10 per cent increase with every minute increase on exposure. The relationship, however, is probably not exactly a linear one. The percentage affected in both sets of experiments following the 8-minute exposure was seemingly too high to fall exactly into such a series. The same perforce is shown by the combined data.

TABLE I

PERCENTAGES AND REGIONS OF E. *dorotocephala* SHOWING AN EFFECT FOLLOWING X-RAY IRRADIATIONS

Minutes Rayed	Total No.	Affected Ant. End		Percentage Frequencies of Regions Showing Effect First		
		No.	Percentage	Anterior Tip	Either Auricle	Margin between Tip and Auricle
2.....	40	5	12.5	80.0	20.0	.....
4.....	40	12	30.0	58.3	33.3	8.3
6.....	40	17	42.5	76.5	11.8	11.8
8.....	40	39	97.5	61.5	38.5	.....
10.....	40	40	100.0	75.0	20.0	5.0
Total rayed.	200	113	56.5	69.0	26.6	4.4

As suspected, the first effect of the rays always appeared at the anterior end of an animal. That at least was true for the one hundred and thirteen animals that were visibly affected in the regular experiments. In one preliminary trial one possible exception occurred. An animal that had been exposed 6 minutes showed a darkening of its tissues back of its head before any effect was visible in the head region. The same animal, however, at a later date showed an X-ray effect at the anterior tip which increased while the darkening back of its head disappeared.

Although the effect always appeared at the anterior end, it was not always the extreme anterior tip that showed it first, but sometimes an auricle and occasionally the margin between an auricle and the tip. Table I gives the percentage frequencies of each region.

Initially the effect always showed up as a gradual darkening of the

tissues in one of the three regions already mentioned. After the 2-minute exposure this darkening effect generally persisted a few days and then disappeared, leaving the animal as normal as it was beforehand, but after the longer exposures the darkened area generally became the starting-point of a wave of disintegration. The level which that wave ultimately reached depended in general upon the dosage that had been given. Following the 4-and 6-minute dosages, it gen-

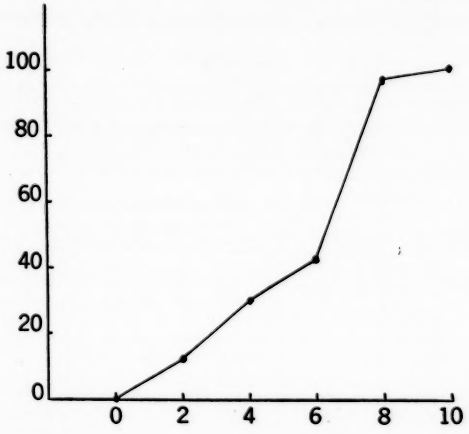

FIG. 1.—Percentages of *E. dorotocephala* showing an effect following five different X-ray dosages. An exposure of 1 minute is equivalent to about 86.4 Roentgen units. Ordinates, percentage affected; abscissae, minutes irradiated.

erally did not pass beyond the head region before it stopped, but after the 8- and 10-minute exposures it generally destroyed all the head tissues and parts of the body region as well. In some cases it continued posteriorly until there were no more tissues to disintegrate. The process from the anterior end, however, never reached the extreme posterior tip, because animals that were destined to disintegrate completely always showed a similar process initiated at the posterior end that traveled anteriorly and met the one from the anterior end somewhere in the middle region, probably posterior to the pharynx, that is, about the level of the zone of fission.

The animals that showed an effect at the posterior end did not all

disintegrate completely. A few survived as short pharyngeal pieces only a few millimeters long.

In order to present the differences in the degree of effect that followed the different dosages, the animals that were affected were divided into three groups: those on which the effect at the anterior end did not extend posterior to the eye level; those that lost all their head tissues but did not disintegrate completely; those that disintegrated completely. The eye level was chosen as a division point because it actually was the limit of the effect following many of the shorter dos-

TABLE II

DEGREE OF EFFECT SHOWN BY *E. dorotocephala* FOLLOWING FIVE DIFFERENT X-RAY DOSAGES; EACH MINUTE OF EXPOSURE IS EQUIVALENT TO APPROXIMATELY 86.4 ROENTGEN UNITS

Minutes Rayed	No.	No. Affected at Ant. End	Percentages Showing the Various Degrees of Effect			Percentages Affected at Post. End
			Eye−	Eye+	Complete Disintegration	
2	40	5	100.0	.....	.....	.....
4	40	12	58.3	41.7	.....	.....
6	40	17	41.2	58.8	.....	.....
8	40	39	12.8	56.4	30.8	61.5
10	40	40	.....	10.0	90.0	100.0

ages. The disintegration in such cases would proceed back to the eye region and then stop, leaving an animal with a blunt anterior margin and two eye spots barely visible on it. If the eye tissue disintegrated, then the process generally continued posteriorily at least several more millimeters. Furthermore, as will be shown in detail later, animals that lost only tissues anterior to the eye level always regenerated normally all lost parts, whereas animals in which the disintegration extended to a region posterior to the eye level seldom did.

Table II gives the percentage of animals following the different dosages that fall in each of the three divisions. It will be seen that all of those that showed an effect after the 2-minute exposure fall in the first group, which in the tables is labeled "Eye−." Those exposed 4

and 6 minutes fall almost in equal numbers into the first two. The 8-minute dosage was the only one to give animals in all three divisions. After the 10-minute exposure all of the animals disintegrated completely except four that persisted as short pharyngeal pieces.

Table II also gives the percentages that showed an effect at the posterior end. It will be seen that such an effect appeared only on animals that had been exposed 8 or 10 minutes, and then, as will be shown later, only after the disintegration from the anterior end had proceeded to a level near the pharynx.

TABLE III

TIME OF APPEARANCE OF EFFECTS ON *E. dorotocephala* FOLLOWING VARIOUS EXPOSURES TO X-RAYS; EACH MINUTE EXPOSURE IS EQUIVALENT TO APPROXIMATELY 86.4 ROENTGEN UNITS

Minutes Rayed	Number of Worms	Anterior End		Posterior End		Completely Disintegrated	
		No.	Mean Day First Effect	No.	Mean Day First Effect	No.	Mean Day
2	40	5	16.8	.....	.....	.....	.....
4	40	12	16.8	.....	.....	.....	.....
6	40	17	18.7	.....	.....	.....	.....
8	40	39	18.4	28	28.1	12	40.2
10	40	40	21.1	40	29.4	36	39.6

In accordance with the findings of other irradiation studies on living tissues, the effect of the X-rays in these experiments was not visible immediately after an exposure but after a latent period of from 10 to 28 days. Table III gives the mean day following the irradiations on which the effect was first apparent at the anterior end, also the mean day for the first appearance of the effect at the posterior end. It will be noticed that the latter appeared on an average of about 10 days later than the former. Although there appears to be an increase in the length of the latent period with an increase in dosage, the trend is probably not a real one. The numbers affected in the case of the shorter dosages are small, and the mean length of the period following the 10-minute exposure is increased primarily by a few exceptionally long ones.

One of the most surprising results was the slow rate at which the disintegration wave or waves traveled over the animals. The mean days for the complete disintegration of animals, following the 8- and 10-minute exposures that are given in Table III, give some idea of this rate. In Figure 2 an actual case is illustrated. An animal that

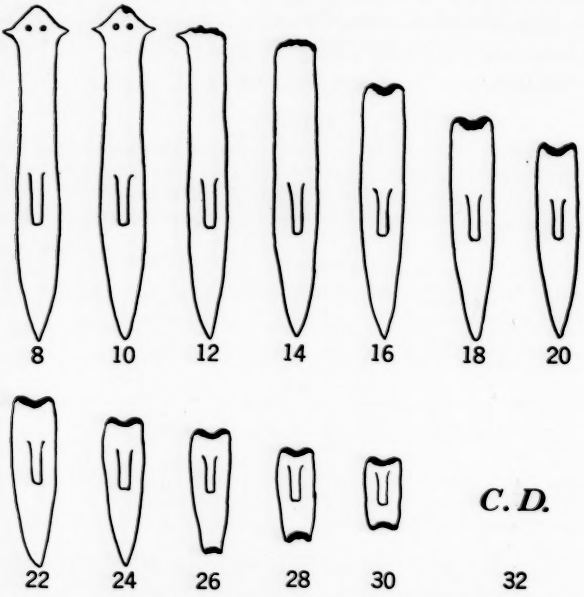

FIG. 2.—The successive stages of disintegration shown by one individual (*E. dorotocephala*) exposed to X-rays 10 minutes, a dosage equivalent to approximately 864 Roentgen units. The numbers below the figures represent days after the irradiation. The letters *CD* stand for "Completely Disintegrated."

was irradiated 10 minutes is shown as it appeared on alternate days after an effect was first apparent. It will be seen that the process from the anterior end in that case required approximately twenty days to pass from the extreme anterior tip to a level near the pharynx, a distance of not more than 10 or 12 mm. The same was true for many other cases. Several estimates indicated that the average rate was less than a millimeter per day.

The illustrations in Figure 2 also show several other points of interest. For instance, they show how completely the tissues already acted upon were lost. In only a few cases was partly disintegrated tissue seen adhering to the animal. The affected margin was generally as smooth or even as one formed by the cut of a sharp knife. The illustrations also show the U-shaped appearance of the affected margin that was presented as the disintegration processes traveled over the body region.

TABLE IV

DEGREE OF REGENERATION SHOWN BY FORTY-ONE INDIVIDUALS (*E. dorotocephala*) WHICH LOST ALL THEIR HEAD TISSUES DUE TO THE EFFECTS OF X-RAYS BUT WHICH DID NOT DISINTEGRATE COMPLETELY; ANIMALS AFFECTED BY 2-MINUTE EXPOSURE AND SOME OTHERS WITH LONGER PERIODS OF IRRADIATION LOST ONLY PORTIONS OF THEIR HEAD TISSUES AND REGENERATED NORMALLY ALL LOST PARTS

Minutes Rayed	No.	Regeneration: Percentages				
		Normal	Teratophthalmic	Teratomorphic	Anophthalmic	Acephalic
4	5	60.0	40.0	.....	.....	.....
6	10	50.0	10.0	10.0	30.0	.....
8	22	40.9	4.6	13.6	9.1	31.8
10	4	.....	.....	.....	.....	100.0

In view of the results of Curtis (1928), regeneration of tissue in *Euplanaria* following X-ray irradiations was hardly expected. However, in these experiments some regeneration followed in almost every case. To express the degree, the conventional terms "anophthalmic," "teratomorphic," "teratophthalmic," and "normal" are adopted. It will be seen from the figures given in Table IV that animals which lost only portions of their head tissues regenerated normally in every case all lost parts, and that those that lost their head tissues, if they did not disintegrate completely, regenerated in most cases some new tissues. It is true that eleven remained acephalic, but even they had the capacity to regenerate, for at a later date when the tissues at their anterior ends were lacerated, they regenerated in every case head tissue of some type. One even went so far as to form a normal head.

The regeneration of tail tissue that followed, although it was in

some cases quite extensive, was not measured with any degree of accuracy. The only statement that can be made regarding it is that it occurred to some extent in almost every case.

In this connection a statement should perhaps be made relative to the occurrence of fission of the animals in the experiment. About 20 per cent of those exposed divided after the irradiation when they were placed individually in Syracuse watch glasses. In all such cases a normal tail regenerated immediately.

Having presented the results on disintegration and regeneration more or less independently, it seemed of interest to give, if possible, a joint picture of those results. To obtain figures for a graph that might do this, every animal was given on alternate days, throughout the course of the experiments, one of four different values. The value of 100 was assigned to an animal that was from all appearances normal, i.e., one that had never been affected or had been affected and had normally regenerated all lost parts. The value of 70 was given to an animal that showed an effect at its anterior end which had not at the time passed the eye level, also to an animal that had previously lost its head tissues but had regenerated at the time a head which was not entirely normal but which contained at least one eye. The value of 30 was given to an animal that had lost all its head tissue and had not at the time regenerated tissues which included eyes or even an eye. And the value of 0 was given to an animal that had disintegrated completely. The arithmetic means of the values for each alternate day for a given lot of animals were then obtained. In Figure 3 they are plotted against time.

The curves obtained show the day on which an effect first appeared on one or more animals in each lot, also the joint effect of the disintegration and the regeneration of a lot as a whole at any given time throughout the course of the experiments. It will be seen that the curve of the lots exposed 2 minutes drops from the normal on the fourteenth day and reaches it again 10 days later. Those for the 4-, 6-, 8-, and 10-minute exposures show increasingly greater effects and less regeneration.

DISCUSSION

As regards the existence in *E. dorotocephala* of a longitudinal gradient of susceptibility to X-rays, there can be no question. As

already shown by the data presented in the tables, the tissues at the anterior end of an animal are affected by X-ray dosages that produce no effect in other regions of the body. Furthermore, an effect produced by a heavy dosage starts at the anterior end of an animal

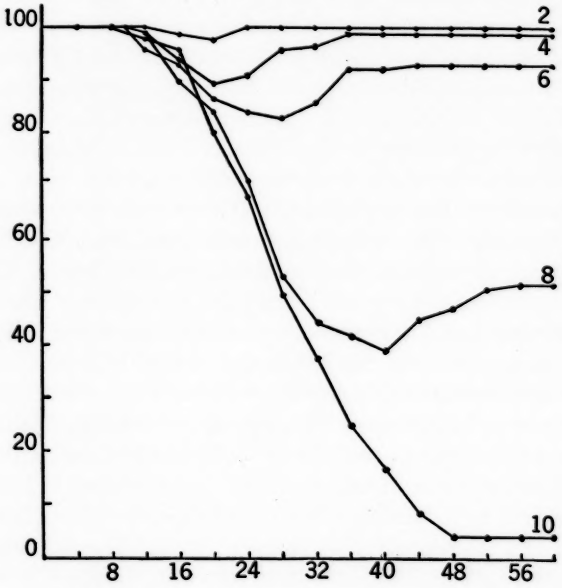

FIG. 3.—A composite picture of the degree of disintegration and regeneration shown by lots of forty individuals of *E. dorotocephala* following exposures to five different dosages of X-rays. Ordinates, arbitrary values. A value of 100 represents no effect or an effect followed by normal regeneration of all parts. A value of 0 represents complete disintegration. The abscissae are days after the irradiations. The numbers on the curves are length of exposures in minutes.

and proceeds posteriorly over the anterior zooid and is met by a similar process which starts at the posterior end and proceeds anteriorly. If time of appearance is taken as a criterion of degree of susceptibility, the gradient observed may be said to have its high point at the anterior end, dip gradually to the pharyngeal region or a level a millimeter or two posterior to the pharynx, and then rise gradually but not very rapidly to the region of growth at the posterior tip.

Having established the existence of a gradient of susceptibility to X-rays, the question might be asked, "What conclusions, if any, can be drawn from that knowledge?" It is well known that X-rays produce changes in many chemicals, more readily in some than in others. Hardy and Willcock in 1903 reported that iodoform dissolved in chloroform liberates free iodine when irradiated. In X-ray photography the silver chloride and silver bromide of photographic emulsions are, of course, reduced. Many other examples could be cited. Thus it appears that the gradient observed in these experiments might be primarily an expression of a differential distribution of a chemical or chemicals along the antero-posterior axis.

X-rays are also known to produce a greater effect on active cells than on cells that are in a more quiescent state. This was shown as early as 1903 by Bergonie and Tribondeau in their classical experiments on the testis of the white rat. They found that actively dividing spermatogonia and spermatocytes are more susceptible to the action of X-rays than are the Sertoli and interstitial cells. The same has been demonstrated in many other animals since then. Actively growing tumors, it is generally agreed, are more susceptible to X-rays than are surrounding tissues. In the preliminary trials of these experiments some evidence was obtained that the same cells will respond differently to X-rays when physiologically active than they will when less so. The digestive tissue of animals starved two weeks showed little or no effect of moderately strong dosages of X-rays, while the same tissues in well-fed animals disintegrated rapidly when exposed to dosages of similar strength. Thus the possibility also seems to exist that the gradient revealed might be primarily an expression of regional differences in cell activity.

In view of the fact that the susceptibility gradient revealed in these experiments is almost identical in pattern with those brought out by many other agents including acids, bases, alkaloids, KCN, chloroform, chlorotone, extremes in temperature, ultra-violet light, etc., as shown by the studies of Child and his students, it seems probable that the latter suggestion is the more nearly correct one. That there exists in *Euplanaria* and many other organisms such an antero-posterior gradient of physiological or metabolic activity is, of course, the conclusion reached by Child many years ago. The results of the

present experiments may, therefore, at least be considered in harmony with his theories if not a substantiation of them.

Although the susceptibility gradient in *Euplanaria* revealed by X-rays is in general in agreement with those revealed by other agents, a few slight differences might be pointed out. Child has found in *Euplanaria* several zones in the region posterior to the pharynx which show a slightly higher susceptibility to certain agents than do neighboring tissues. These he has interpreted to be the anterior ends of zooids in formation. With a weak solution of KCN he was able to bring out as many as three such zones in large animals (Child, 1930). In the present experiments such regions were not observed. It is possible that they were overlooked, although this does not seem probable. Another possibility is that the slight differences in susceptibility which indicate the zones were reduced by the prolonged starvation or obliterated by an effect of the rays during the long period that intervened between the irradiation and the disintegration.

With respect to the existence in *Euplanaria* of a medio-lateral susceptibility gradient to X-rays, no very definite conclusion can be reached from the data obtained. It is true that the disintegration, as it progressed over the body regions, appeared to be farther advanced in the median line than it was at the lateral margins, but how much this effect was due to a differential susceptibility and how much it was due to muscular contraction cannot be stated with any degree of certainty. An animal cut transversely with a sharp knife will show almost as strikingly a U-shaped margin.

No indication of a dorso-ventral gradient was obtained from the regular experiments involving starved animals. In preliminary trials it was noticed that well-fed animals exposed to X-rays showed lesions on the dorsal side along the course of the three branches of the gastrovascular cavity. This might be interpreted as an indication of a dorso-ventral gradient, but it is more likely an expression of differences in thickness or toughness of the tissues on the dorsal and ventral sides. Allee (1928) reported similar lesions on the dorsal side of well-fed *Planaria* exposed to ultra-violet light.

The practically identical length of latent periods, observed in the present experiments, following a 2- and a 10-minute exposure was

scarcely expected. It had been thought likely that the action of a 10-minute dosage would bring an effect on a given tissue sooner than would the action of a 2-minute dosage. This idea was entertained primarily on the basis of the results obtained by the writer in X-ray experiments with guinea pigs (Strandskov, 1932). In those experiments a longer exposure to X-rays brought on a sterility of male guinea pigs sooner than did shorter ones. In these experiments, as shown in Table III, the trend was actually reversed, but, as already pointed out, the differences in the means obtained are probably not significant.

The degree of regeneration of tissues that followed the disintegration of parts due to the action of X-rays was perhaps the most surprising of all the results obtained. Curtis in 1928 reported that several species of flatworm, *Planaria agilis* and *Planaria maculata*, fail to regenerate new tissue following X-ray irradiations, when a "sufficient exposure" is used. In the present experiments some regeneration of tissues followed in almost every case, even in animals that lost the anterior third of their body as the result of disintegration due to X-rays. It is true that some short pieces remained acephalic, but even short pieces of unirradiated animals may fail to regenerate new tissue. Curtis is of the opinion that regeneration of new tissues is dependent upon what he calls "formative cells," and that the reason why irradiated animals do not regenerate is that X-rays destroy these cells before they destroy others. Such cells it is true, according to the laws of Beronie and Tribondeau, would be the first to be destroyed if they were present. Whether or not "formative cells" of the type described by Curtis are present in *E. dorotocephala* the writer cannot state, but if they are, they do not seem to be especially susceptible to X-rays, at least not to the kind emitted by the tube under the conditions given.

SUMMARY

1. Five lots of forty animals each of *E. dorotocephala* were exposed to X-ray dosages ranging from 172 Roentgen units to 864.

2. One hundred and thirteen animals of the two hundred irradiated showed an effect of the rays.

3. The degree of the effect varied in general with the length of the exposure.

4. The effects revealed an antero-posterior gradient of susceptibility to X-rays similar in pattern to the susceptibility gradients brought out by other agents in the same animal.

5. This appears to be in harmony with the idea of Child that there exists in *Euplanaria* and many other animals an antero-posterior gradient of physiological or metabolic activity.

6. Although unexpected on the basis of the results of Curtis, the affected animals that survived, regenerated in most cases some, if not all, the tissues that they had lost as the result of the effects of the X-rays.

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THYROXIN AND REGENERATION IN THE HYDROID PENNARIA

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EARLIER studies of thyroid function have already shown that the thyroid derivative, thyroxin, exerts a depressing effect on cell division and on differentiation, in such diverse organisms as *Paramecium* (Torrey, Riddle, and Brodie, 1925), the sea urchin *Echinometra*, and the ascidian *Phallusia* (Torrey, 1928). The probability that chick fibroblasts respond similarly to thyroxin has also been indicated. The present evidence from *Pennaria* gives added support to the general view that thyroxin may properly be regarded as a depressant of cell division throughout the animal kingdom. Like the larvae of the urchin and ascidian, *Pennaria* is a metazoan, simpler, but lacking, as they do at all stages in their development, those endocrines that are especially characteristic of the vertebrates. Accordingly, the effects of thyroxin can be followed in the course of regenerative development with a certain directness which experiments on vertebrates, with their more complex organizations and interacting endocrines, do not permit.

Pennaria tiarella is a very showy species, readily recognized from the collector's boat in the shallow waters over the shore reefs at the Dry Tortugas,[1] clinging in feathery tufts to old upstanding stalks of alcyonarian corals. From a single holdfast many dark stems rise in broad, graceful arcs to lengths that approximate 200 mm. in the longest. Each stem carries two regular sub-opposite ranks of slender, alternating branches that spring away from it at a sharp angle and carry, each on its convex aspect, a row of white polyps on short pedicels. The stem is naked for about the lowermost third. The branches vary gradually and regularly in length. At the proximal end of the series, they vary from a few to 20 mm. long. In the middle third of the stem they reach their maximum length, which may be

[1] My work was done while a guest at the Tortugas Laboratory of the Carnegie Institution.

50 mm. Thence, they decline gradually to the budding zone at the distal end.

In the experiments reported below, stems only were used, segments of appropriate length being cut from their distal thirds and the branches removed close to the stems, a short basal stump being left in each case. Regeneration was manifested by new growth and differentiation at the various wound surfaces which involved proximal and distal ends of each stem segment as well as stumps of branches. There was considerable variation in amount and position of this new growth. Some stems produced hydranths at every possible point; while others, similar to them in size and general appearance, produced none anywhere. It was not always possible to obtain for a given experiment stem segments that were strictly uniform in size, age, and general condition. In such cases the attempt was made to apportion them to thyroxin and control dishes, so as to afford comparable distributions of the variations. Certain irregularities that occur in the tables may be referable, in part at least, to these variations in material. They were not sufficient to affect materially the conclusion that the regeneration of *Pennaria*, under the conditions of the experiments, was retarded by thyroxin, the effect varying, as in the case of cell division, though less precisely, with the concentration.

The regenerating stems were kept in finger bowls containing 100 cc. sea water with an initial pH of 8.0–8.4. The bowls were arranged in pairs, each experimental bowl with its control, the former containing thyroxin (Squibb), freshly prepared in appropriate dilution. Solutions of thyroxin were made by dissolving a few milligrams of the crystals in 2–4 drops of N/10 NaOH, transferring this solution by pipette to a few cubic centimeters of distilled water to which a drop of N/10 NaOH had been added, and mixing the latter with sea water to the dilution required. In dilutions of 1:50,000 and 1:66,000, in sea water, precipitation frequently occurred; in dilutions of 1:100,000 also, but much more rarely. It did not occur in distilled water, or in Van t'Hoff's solution. The precipitate did not redissolve on the addition of sea water, acids, or alkalis. It appeared to be dependent on the presence of organic substances in solution in the sea water used, and it was associated with diminished physiological potency of

the solutions in which it occurred, as will be pointed out below. It was concluded that the precipitate, which was not otherwise identified, was composed of thyroxin in part at least.[2]

This precipitate occurred in a 1:50,000 solution in the experiment the results of which are summarized in Table I. Comparing this culture with the control, it will be seen that hydranth regeneration had proceeded just as though thyroxin had not been present at all; while regeneration in each of the other cultures was definitely inhibited in amounts varying with considerable regularity, inversely as the thyroxin in solution.

TABLE I

EFFECT OF THYROXIN ON REGENERATION OF *Pennaria*

CULTURES	NO. OF STEMS	NO. OF WOUND SURFACES	NO. OF HYDRANTHS AT 22 HOURS			
			Large	Small	Buds	
A. Thyroxin in concentrations of 1:50,000 (precipitation)	17	222	15	3	several	on 6 stems
1:100,000....	17	218	.....	1	3	on 2 stems
1:200,000....	16	207	3	5	3	on 6 stems
1:400,000....	18	233	2	5	3	on 2 stems
1:800,000....	17	222	5	6	3	on 5 stems
B. Control.....	17	219	15	4	12	on 7 stems

That thyroxin in a concentration of 1:200,000 retards hydranth regeneration appears from Table II, which gives the results of four experiments. In each case, the hydranths in the controls far outnumber those in the thyroxin cultures.

In another experiment, concentrations from 1:200,000 to 1:1,000,000 were used (Table III). Here the seven columns indicate, in succession from left to right, numbers of stems, numbers of wound surfaces, hydranths at 16 hours, hydranths at 24 hours, holdfasts at 24 hours, ratios of hydranths at 24 hours to wound surfaces, ratios of the sum of hydranths and holdfasts at 24 hours to wound surfaces. The inclusion of holdfasts does not materially change the results already obtained for hydranths alone. The low H/W ratio for the

[2] A similar precipitate was noted by Carter (1931).

1:1,000,000 culture appears to be exceptional, to judge from the fact that this was the only culture in which no hydranths were produced

TABLE II

EFFECT OF THYROXIN OF REGENERATION OF *Pennaria*

Cultures	No. of Stems	No. of Wound Surfaces	No. of Hydranths			Time (Hours)
			Large	Small	Buds	
A. Thyroxin in concentration of 1:200,000	17	205	5	3	.....	18
Control	17	214	7	24	.....	18
B. Thyroxin in concentration of 1:200,000	19	248	1	.....	.....	23
Control	19	235	30	.....	.....	23
C. Thyroxin in concentration of 1:200,000	16	207	3	5	3	22
Control	17	219	15	4	12	22
D. Thyroxin in concentration of 1:200,000	18	177	4	5	.....	22
Control	18	187	30	6	.....	22

TABLE III

EFFECT OF THYROXIN ON REGENERATION OF *Pennaria*

Cultures	No. of Stems	No. of Wound Surfaces	No. of Hydranths		No. of Hold-fasts at 24 Hours	H/W	Hhf/W
			At 16 Hours	At 24 Hours			
Thyroxin in concentrations of:							
1:200,000	42	227	35	39	23	0.171	0.273
1:400,000	39	216	23	34	24	0.157	0.268
1:600,000	42	226	29	46	16	0.203	0.283
1:800,000	40	218	38	45	20	0.206	0.298
1:1,000,000	19	107	17	17	13	0.159	0.280
1:1,400,000	20	104	26	33	12	0.317	0.432
Control	43	226	57	94	21	0.411	0.508

between the sixteenth and the twenty-fourth hour after the experiment began. There is a general increase in the amount of regeneration as the concentration of thyroxin decreases, with the maximum

in the 1:1,400,000 culture where, however, it does not yet equal the control.

In an experiment in which the addition of thyroxin was delayed 8 hours after the stems were prepared (Table IV), there was no sig-

TABLE IV

REGENERATION OF *Pennaria* IN THYROXIN ADDED 8 HOURS AFTER CUTTING

CULTURES	NO. OF STEMS	NO. OF WOUND SURFACES	HYDRANTHS AT 19 HOURS			HYDRANTHS AT 26 HOURS		
			Large	Small	H/W	Large	Small	H/W
Thyroxin in concentration of 1:200,000	18	182	14	25	0.214	49	5	0.247
Control	18	185	38	29	0.362	79	8	0.470

nificant departure from the result typically obtained when the stems were exposed to thyroxin throughout the course of the experiment.

When, however, this procedure was reversed to some extent by exposing the experimental stems to thyroxin for the first 4 hours

TABLE V

EFFECT OF THYROXIN ON REGENERATION OF *Pennaria* WHEN PRESENT DURING FIRST 4 HOURS ONLY

A. Thyroxin culture:
   0 hour, 26 stems in sea water containing thyroxin 1:200,000
   4 hours, transferred to sea water without thyroxin
   18 hours, 21 stems (5 in poor condition and discarded) with 240 wound surfaces, 86 hydranths; H/W 0.358

B. Control:
   0 hour, 29 stems in sea water
   4 hours, transferred to new sea water
   18 hours, 20 stems (9 in poor condition and discarded) with 228 wound surfaces, 70 hydranths; H/W 0.307

only, regeneration in the thyroxin cultures exceeded that in the controls (Table V). It would seem as though the brief application of thyroxin had actually accelerated hydranth regeneration. For this exceptional result, however, there may be an alternative interpretation, namely, that the thyroxin was applied for too brief a period to

produce any effect upon the thyroxin cultures, which accordingly regenerated like controls. In support of this view it may be said that while the figures for the thyroxin culture are well within the range of variation of the controls in several other experiments under similar conditions (e.g., Table IV), the control is near the lower limit of this range. But my data in this connection are too few to demonstrate the status of this apparent exception, which must await further experiments.

TABLE VI

EFFECTS OF THYROXIN, POTASSIUM IODIDE, AND METALLIC IODINE ON REGENERATION OF *Pennaria*

CULTURES	NO. OF STEMS	NO. OF HYDRANTHS				
		18 Hours		26 Hours		
		Small	Buds	Large	Small	Buds
A. Thyroxin in concentration of 1:100,000	14	.....	5	9	4	0
B. Potassium iodide comparable as to iodine with thyroxin 1:100,000	14	12	8	16	4	0
C. Iodine comparable as to Iodine with B	14	.....	7	1	6	0
D. Control	14	9	9	15	7	0

Finally, the effects of iodine in three forms on hydranth regeneration were observed in an experiment the results of which are embodied in Table VI. The stems were of approximately equal length, each with approximately the same number of wound surfaces. Metallic iodine proved more depressing than thyroxin, while potassium iodide had no appreciable effect.

In seeking an interpretation for the depressing effects of thyroxin on cell division and differentiation that have been shown to occur in various organisms, the latest being the hydroid *Pennaria*, one is at once struck by the fact that thyroxin has a well-established reputation as an accelerator of oxidative metabolism in the higher vertebrates. To assume the same rôle for thyroxin among the invertebrates with which we are at present concerned would force us to the

conclusion that the reactions on which cell division and development depend are not primarily oxidative in type. And with this conclusion our recent results with dinitrophenol are in essential accord (Torrey, 1933). As yet, however, no direct determinations of the influence of thyroxin on oxygen consumption have been made for any of the organisms used in our experiments. In the absence of such desirable data, I do not feel warranted in pressing a point about which differences of opinion already exist that can be composed only with the help of crucial observations.

SUMMARY

The essential processes underlying the regeneration of the hydranth in the hydroid *Pennaria* were depressed by solutions of thyroxin in concentrations of 1:50,000 to 1:1,400,000, in general directly with the concentration.

Metallic iodine was a more effective depressant of regeneration than thyroxin. Potassium iodide had no appreciable effect.

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HYDROGEN ION ACTIVITY CHANGES IN GALLERIA MELLONELLA DURING METAMORPHOSIS, AS DETERMINED BY A GLASS ELECTRODE WITH MICRO-VESSEL

(One figure)

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THIS paper deals with the hydrogen ion activities of the body fluids of *Galleria mellonella* (bee moth) during late larval life and throughout pupal development. The glass electrode was selected for the measurements, since it was found to be more convenient and accurate than the hydrogen and quinhydrone electrodes for this type of work. This investigation is a part of a series of studies on the physiological and biochemical processes occurring during the metamorphosis of *G. mellonella*.

The body fluids of insects are very frequently on the acid side of neutral, although sometimes they are slightly alkaline. This is indicated by the work of Jameson and Atkins (1921), Crozier (1923–24), Bodine (1925, 1926), Brecher (1925), Fink (1925), Glaser (1925), Ludwig (1933), and others. Some of these investigators employed the colorimetric method, while others made use of the hydrogen electrode. Few investigations have been made regarding the pH of body fluids at the different stages of metamorphosis. Fink (1925) reported for several species that a change occurs during prepupal and early pupal development from a neutral to an acid reaction (pH 6.8 to 5.9) and that, as metamorphosis is completed, a converse change occurs. Ludwig (1933) has found that in the case of the Japanese beetle the pH of the blood changed from an average of 7.07 in the third instar larva to 6.79 in the late prepupae. When pupation occurred, he observed a change to a more alkaline condition, but there was no significant change during the pupal stage. He suggests that the increase in acidity of the late prepupae may be due to the

autolysis of larval tissue. Bishop (1923–24) has suggested that an increase in the acidity of the tissues of the honey bee may be one factor favoring histolysis. Dernby (1918) has shown that the autolysis of tissue of the liver and pancreas proceeds furthest in a range of pH between 5 and 6. He attributes this autolysis to the action of pepsin and trypsin, and he states that when the pH is such that these enzymes can act simultaneously the autolysis is most rapid and complete. It is pointed out by Dernby that, when different tissues autolyze, it is impossible to predict in what direction the pH will change, since this depends upon the chemical constituents of the different tissues. Brecher (1925) and Glaser (1925) in investigations on the body fluids of several species of insects found no changes that could be correlated with metamorphosis. From the work of Bishop (1923–24) on the honey bee there appears to be a relationship between the CO_2 tension and the pH of the blood. He found in the larvae and pupae that a decrease in pH is associated with an increase in the CO_2 tension. Evidence that this is also probably true for *Galleria mellonella* will be mentioned later.

MATERIAL AND METHODS

Measurements of the hydrogen ion activities were made on the body fluids of 63 larvae and 420 pupae of *Galleria mellonella* by means of a glass electrode assembly employing a vacuum-tube amplifier as described by Hill (1931), a Leeds and Northrup Type K potentiometer, and a galvanometer of high sensitivity. The microvessel used with the glass electrode was of the closed type described by Taylor and Birnie (1933), and it required only a drop of fluid in filling. The measurements were made with the glass electrode, calomel electrode, and animals maintained at a temperature of $30 \pm 0.5°$ C. in an electrically shielded, thermostatically controlled air chamber. After each measurement the performance of the glass electrode was checked with a standard buffer solution.

The animals used were kept throughout life at 30° C. and were segregated at a suitable time in advance of the measurements, so that the age of each animal in larval or pupal life could be closely determined. A single individual was employed for each measurement. In filling the glass electrode chamber, the end of the animal was cut off with a pair of fine scissors, and the cut end was quickly

placed in the funnel-shaped inlet. The body was squeezed, which caused the fluid contents of the animal to pass into the chamber and fill it. After a small amount of fluid began to pass into the outlet tube, the chamber was closed, preventing the exchange of gases between the sample and the outside air. Sufficient body fluid readily to fill the fluid chamber of the glass electrode vessel was obtained easily from a single larva and from a single pupa up to about the last quarter of pupal development. The body fluid flowed easily into

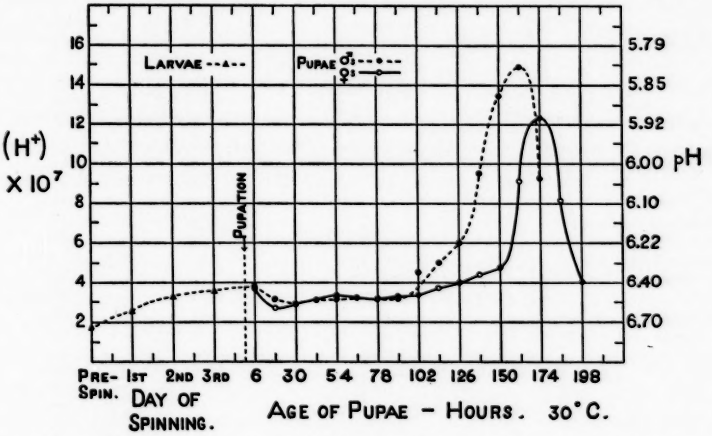

FIG. 1.—Hydrogen ion activities of body fluid during metamorphosis. Temperature 30° C.

the chamber; it contained some cells and fragments of tissue in suspension; the amounts of these were considerably greater in the case of pupae in the last quarter of the pupal period. This probably accounts for the fact that the potentials were less steady when pupae near the end of their development were used. It is believed that the individual measurements made on the larvae and on pupae before the last quarter of the pupal stage are accurate to within 0.02 pH and that the accuracy is slightly less for measurements on pupae older than these.

RESULTS

The results of the hydrogen ion activity measurements are shown in Figure 1. The curves are plotted on a hydrogen ion activity (ap-

parent concentration) scale, but corresponding pH values are shown at the right. It was felt that this would give a clearer picture than if a logarithmic (pH) scale were used. Each point on the curves represents the average value for a number of animals. The pH values are all on the acid side of neutral. As it is not possible to dis-

TABLE I

NUMBER OF ANIMALS USED AT THE VARIOUS AGES

Larvae		Pupae		
Age	Number of Animals Used Individually (Both Sexes)	Age (Hours)	Number of Males Used Individually	Number of Females Used Individually
Pre-spinners	17	6	12	27
		18	10	20
		30	12	19
First-day spinners	17	42	9	16
		54	14	20
Second-day spinners	23	66	17	15
		78	12	19
		90	11	14
Third-day spinners	6	102	11	26
		114	6	14
		126	12	13
		138	9	10
		150	7	8
		162	18	10
		174	2	19
		186	.....	10
		198	.....	2
Total	63		158	262

tinguish between the sexes in the larval stage, the curve for animals in this stage is for both sexes. The values for larvae are for the last few days of larval life, which at 30° C. is of approximately 25 days' duration. Separate curves are given for male and female pupae throughout their development.

The number of larvae or pupae used individually in establishing each point on the curves is shown in Table I. The larvae designated "pre-spinners" were mature animals which had stopped feeding, had come to the top of the culture jars, and were on the verge of active spinning.

The curve for the larvae indicates that, as the time of pupation approaches, the hydrogen ion activity of the body fluids increases. The curves for male and female pupae show that the hydrogen ion activity drops slightly at first, remains fairly constant until about the middle of pupal development, then increases markedly, and finally decreases. The divergence of the curve of one sex from that of the other sex in the last half of pupal development is due, at least in part, to the fact that the males have a shorter developmental time at this temperature than the females. The maximum part of the curve for the males reaches a higher level than the corresponding part of the curve for the female pupae, but this difference is without statistical significance. The rise of the two curves for pupae near the end of pupal development above the preceding low levels has been shown to be statistically significant, and the fact that the points on the curves fall in orderly sequence adds further significance.

DISCUSSION

Other investigators, including Bodine (1926), Fink (1925), and Ludwig (1933), have reported pH values on the acid side of neutral for the body fluids of insects. In some insects the fluids from different parts of the body may have different pH values, as found by Bodine (1925) for certain Orthoptera. Although, in the work here reported, no measurements were made on fluids from different parts of the bodies of larvae or pupae, it is likely that in the bee moth the body fluids from different parts do not have the same pH values. In making a filling of the electrode vessel, most of the body fluid from one animal was required and subjected to a certain amount of mixing, so that the pH value obtained for a single animal probably approaches an average value for the various parts.

The hydrogen ion activity curves reported here for pupae throughout development approximately parallel the oxygen consumption and carbon dioxide production curves reported by Taylor and Steinbach (1931) for the same animal and at the same temperature. This suggests that perhaps the changes in pH of the body fluids are mainly reflections of the variations in CO_2 tension which one would expect would accompany changes in CO_2 output. This idea is in harmony with the findings of Bishop (1923–24) already mentioned.

It is possible that the decrease in pH before pupation may, in some way, initiate pupation and histolysis; but, on the other hand, it and the other pH changes during metamorphosis may be the result of the biochemical changes accompanying these processes. Other plausible explanations of the changes in the hydrogen ion activity of the body fluids of *Galleria mellonella* during metamorphosis might be offered, but it seems premature to suggest them at this time in view of the many gaps in our knowledge of the physiology of animals during metamorphosis.

SUMMARY AND CONCLUSIONS

1. Measurements of the hydrogen ion activities of the body fluids of *Galleria mellonella* have been made throughout metamorphosis, using a single animal for each determination. A sensitive glass electrode assembly was employed. At all stages the body fluids were found to be on the acid side of pH 7.0.

2. Late in larval development the fluids become more acid as pupation approaches. Immediately after pupation, there is a slight decline in hydrogen ion activity which is not statistically significant; the level then remains fairly constant for both sexes until pupal development is about half completed. At this point there is a significant and marked increase in hydrogen ion activity in both sexes, the increase for the males coming earlier than that for the females. The hydrogen ion activity for both sexes then decreases just before the time of emergence. The curves for hydrogen ion activity approximately parallel the curves for CO_2 production in the same species at the same temperature.

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THE ORIGIN AND DEVELOPMENT OF REFLEXES IN THE CAT DURING THE THIRD FETAL WEEK

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A STUDY of development of somatic motility in the cat was begun in this laboratory in 1929. Observations have accumulated slowly because there are many difficulties besetting experiments on normal, living cat embryos and fetuses. Not the least of these is a limited supply of satisfactory material occasioned by infrequent breeding periods. Some of the results of earlier investigations have been reported (Windle and Griffin, 1931; Windle, O'Donnell, and Glasshagle, 1933). During the past season attention has been focused on an analysis of some of the earliest responses to various types of stimulation applied to cat embryos and young fetuses. To the earlier studies, we wish to add observations on twenty new litters listed in Table I.

Throughout the course of these investigations every effort has been made to observe phenomena objectively, to record factual observations, to attempt no premature interpretations and correlations, and to prevent existing theories of behavioral development from coloring our observations. It is especially important to avoid speculation regarding interrelationship of early embryonic movements before sufficient observations have been made. Although we are uncertain regarding many points in the development of somatic behavior, a few seem to be sufficiently clear to be described in the hope that other investigators will be aided thereby. Our conclusions should be regarded tentative, and no final account of the development of behavior should be formulated upon them until more detailed experiments have been performed.

METHODS

Anemic decerebration (Pollock and Davis, 1924) of the pregnant animals was the routine procedure an hour or more before observa-

[1] Contribution No. 204.

tions on the embryos was begun. This was done in order that an anesthetic might be avoided during the study of fetal movements. There is reason to believe that sufficient ether to maintain an experimental animal in the quiescent state may seriously interfere with the reactions of fetuses in the uterus.

TABLE I

MATERIAL COLLECTED IN 1933 ARRANGED ACCORDING TO AGE OF LITTERS

Litter No.	No. of Embryos	Days and Hours since Mating*	Size in Mm.	Time Allowed for Recovery from Ether Anesthetic
7:33	4	23 d.	11.0–12.5	1 hour 15 minutes
6:33	4	23 d., 2 h.	11–12	1 hour 10 minutes
4:33	4	23 d., 3 h.	8.5†–13.0	‡
8:33	1	23 d., 6 h.±	13+	1 hour 25 minutes
9:33	6	23 d., 10 h.±	12.0–13.5	1 hour 30 minutes
3:33	4	?	14.0–14.5	1 hour 50 minutes
10:33	6	24 d., 1 h.	14– to 15	1 hour 5 minutes
20:33	5	24 d., 2 h.	14–15	1 hour 30 minutes
5:33	4	?	15.5–16.0	1 hour 30 minutes
2:33	5	?**	16	2 hours
15:33	5	25 d.††	12.5–14.5	1 hour 5 minutes
13:33	4	25 d., 2 h.	16.0–17.5	1 hour 15 minutes
19:33	3	25 d., 6 h.	16.0–16.5	1 hour 40 minutes
18:33	3	25 d., 21 h.	16– to 16.5	1 hour 55 minutes
11:33	3	26 d., 5 h.	18+ to 18.5	1 hour 50 minutes
14:33	3	26 d., 5 h.	17.5–18.5	1 hour 35 minutes
17:33	6	27 d., 4 h.	20–22	1 hour 30 minutes
16:33	4	28 d.±§	20.5–22.0	1 hour 25 minutes
12:33	4	28 d., 23 h.	22.0+ to 23.5	1 hour 55 minutes
1:33	5	?	23–26	1 hour 35 minutes

* Several matings were usually permitted. Ages date from the first one determined by vaginal smear.

† One small specimen, probably litter "runt."

‡ Animal died while we were ligating the carotid arteries: cardiac fibrillation for no apparent reason.

** This animal was in heat when delivered to us. It mated immediately, and the embryos should be 23½ days old. However, it is probable that mating first took place before we saw the cat.

†† The size of the embryos of this litter raises a question of accuracy of the age record. We have no evidence that a later mating could have occurred, because the cat was isolated after the first one. Although the embryos may have been curved more than usual, and consequently comparable to slightly larger specimens, there can be no doubt that they were less advanced than normal 25-day specimens.

§ Mating was allowed on two consecutive days. If the second was the fruitful one, the age should be 27± days.

The methods employed in studying embryos and young fetuses have varied according to the nature of the observations planned. As a general rule, the uterus was opened one vesicle at a time and a preliminary examination of each specimen was made. The movements observed and the reflexes elicited at such times are unquestionably as representative of the normal capabilities of the embryo as can be ob-

tained with the present technique. The maternal circulation was perfect, the placenta was intact, the embryo was warm, and was still incased in fluid within the amnion. Surely, observations made during the first few moments after opening the uterus were as nearly free from effects of anoxemia, cooling, drying, fatigue, etc., as can be expected.

After such preliminary examinations, specimens were often removed with placenta attached or else the umbilical cord was simply tied and severed; they were placed in physiological saline solution in a constant-temperature bath. The effects of such procedures were studied, and the movements and reflexes so obtained were compared with those observed during the initial period. Many variations were brought into these procedures of investigation, and almost every one brought forth interesting results.

Several methods of stimulation were employed to elicit responses from the embryos and fetuses. Mechanical agents used were fiber and metal needles, glass rods, probes, or other blunt instruments, and soft brushes. With these, light punctate stimuli, which will be termed "tactile," or coarse "pressure" stimuli resulting from irritation of broader areas or from displacement of parts (flipping limbs or tail) were produced. Often, responses could be obtained by tapping on the amnion and thus transmitting pressure through the amniotic fluid. Electrical stimulation was applied in the form of faradic current from an inductorium whose secondary coil was withdrawn 5–9 cm.; fine nichrome-wire electrodes, spaced half a millimeter apart, were used. Obviously, the age and size of litters and the size of individual specimens played a part in determining the nature and extent of the experiments performed on the cat embryos.

Two or three observers nearly always took part in the experiments, and a stenographer was usually present to take shorthand notes. More than once, overenthusiasm was checked by the conservative judgment of one or another observer; and often valuable suggestions were made in these group studies that would not have occurred to a single investigator.

OBSERVATIONS AND DISCUSSION

It is proposed to describe and discuss a few of the somatic movements that can be elicited from cat embryos and fetuses during the

third fetal week. No attempt will be made to cover all the reactions that can be obtained in this period, nor will detailed protocols be given. A list of the most constantly observed reactions and the size of specimens in which they first appeared, together with the type of

TABLE II

THE ORDER OF APPEARANCE OF CERTAIN CONSTANT RESPONSES IN CAT EMBRYOS

The adequate stimulus is indicated by "F" (faradic shock), "P" (pressure), and "T" (touch).

Size of Specimen in Mm. C.R.L. (Greatest Length)	11	12	13	14	15	16	17	18	19	20
Direct stimulation muscle—shoulder and trunk	F									
Direct stimulation muscle—intrinsic forelimb			F							
Direct stimulation muscle—neck			F							
Direct stimulation muscle—tail			F							
Direct stimulation muscle—intrinsic hind limb				F						
Abduction caudad forelimb—forelimb stimulation		P		F	T					
Head extension—snout stimulation			F	PT						
Contralateral head flexion—snout and jaw stimulation			F				T			
Flexion at elbow—forelimb stimulation				?P		PFT				
Homolateral head flexion—eye stimulation					F		P	T		
Head extension—forelimb stimulation					P					
Passive forelimb movement—snout stimulation					F		P	T		
Abduction hind limb—hind-limb stimulation					P					
Abduction caudad forelimbs—snout stimulation						F	P			
Local flexion pelvis—tapping amnion						P				
Homolateral head flexion—snout and jaw stimulation						F				T
Dorsal flexion wrist—stimulation tips of digits							P	T		
Lateral tail flexion—tapping amnion								P		
Ventral flexion wrist—dorsal stimulation digits								F		
Knee flexion—hind-limb stimulation									P	
Dorsal tail extension—genital stimulation									F	

stimulation which elicited them, will be found in Table II. This is based on new observations, to a large extent; but consideration has been given, in part, to earlier studies which have been reanalyzed.

DIRECT STIMULATION OF MUSCLE

Before any spontaneous movements can be observed, and prior to the appearance of reflexes, the skeletal muscle of cat embryos is

capable of direct response to stimulation. Faradic shocks of various intensities were applied to the surface over muscle masses or the electrodes were plunged into the substance of the embryos. Sometimes direct stimulation was effected by pinching or exerting strong pressure on the embryonic muscles.

The reactions which follow these types of stimulation have characteristics which differentiate them from the first reflex movements of cat embryos, namely: (1) each contraction is relatively slow; (2) it seems to be maximal; (3) it follows the application of a stimulus after no visible latent period; (4) the muscle relaxes very slowly after contracting, and consequently the reaction appears to be tetanic; (5) it can be obtained successively (granting that the stimulus is applied properly); (6) it is localized, and the plane of movement varies closely with the position of the electrodes; (7) it makes little difference whether the embryo is alive or has recently died, nor does its presence depend greatly upon the physiological condition of the parent cat.

The smallest embryo in which muscle contractions could be induced was one 11 mm. long and 23 days old (copulation age). In this specimen, very feeble (and possibly questionable) movements of the forelimb bud and similar contractions of the lateral trunk muscle just caudal to the forelimb followed the application of mild faradic shocks. Other specimens, 11.5–12.0 mm., showed these contractions more clearly. When the stimulus was applied to the forelimb at its attachment, the limb moved outward; when applied to the lateral body wall, there followed a slight lateral flexion of the trunk. The former movement was the more pronounced. We did not obtain contractions of neck muscles in embryos shorter than 13 mm. Perhaps a mechanical factor of head weight entered there. Contractions of the tail muscles occurred in one slightly more than 13 mm. long. The first definite contractions of the muscles which move the hind limb were not encountered in specimens shorter than 14 mm.

The range, strength, and speed of muscle contractions increases directly with the size of embryos. The 13 mm. ones responded to stimulation of the forelimb with a greater number of movements than did smaller specimens. The limb could be made to move forward, outward, or backward at the shoulder, depending upon the

position of the electrodes. Local movement of the distal part of the limb followed stimulation at the elbow.

One may conclude that the first movements elicited from cat embryos are myogenic contractions. This is in accord with observations on a number of forms of animals. (Lewis, 1920; Tracy, 1926; Coghill, 1929; Angulo, 1933*b*; Prosser, 1933).

THE APPEARANCE OF FORELIMB REFLEXES

The first reflex movements of cat embryos have been observed in the early hours of the twenty-fourth day after mating in specimens 13 mm. long (one 12 mm.). They occur in the forelimb and in the neck. Those of the forelimb are of more frequent occurrence and possibly precede those of the neck, in time of development. They are so fleeting and occur so quickly (with an element of surprise) that only by observing a number of them can one be certain of their nature. At the time of a previous report, we lacked sufficient confidence in our observations to say that these foreleg movements are reflexes, and we referred to them noncommittally as "induced movements" (Windle, O'Donnell, and Glasshagle, 1933). Supplied with additional physiological experiments and anatomical studies, we now are prepared to call them "reflexes."[2] The movements of the head are certainly reflexes, because they follow stimulation of the snout, a point some distance from the location of the contracting muscle. Those of the forelimb have all the characteristics of the head reflexes and do not share those of movements produced by direct stimulation of muscle. Our observations indicate that (1) the contraction is quick; (2) it is not necessarily maximal; (3) it usually follows the application of a stimulus after a just-perceptible latent period; (4) the muscle relaxes very quickly after it contracts, the response being a jerk with no tetanic qualities; (5) it cannot be obtained successively in the small embryos—one response seems to render a closely succeeding stimulus ineffectual (a long refractory period); (6) the position of stimulation does not mold the response—earliest movements are always alike (abduction outward and backward); (7) the embryo must not only be alive, but the mother must

[2] We imply the probability that an impulse traverses a sensory neurone, a nervous center in the spinal cord, and a motor neurone to the muscle.

be in perfectly normal condition—even then, early foreleg reflexes are observed only in the initial few moments of the observation period. It will be seen that these characteristics are directly opposite to those of myogenic contractions, and this fact alone should suggest very strongly that the movements of which we write are reflexes. But other evidence supports the conclusion; it has recently been proved that the last links in the first primitive reflex arcs are completed in the spinal cord, especially in the brachial segments of the spinal cord, at the time the first quick movements can be obtained. The number and length of collateral fibers passing into the mantle layer of the spinal cord from the sensory funiculus bears a direct relationship to the presence or absence of these movements (Windle, 1934).

The first reflex movements of the foreleg can be induced by pressure before faradic shocks or tactile stimuli will elicit them. Thus, they occur in 13–14 mm. embryos when the amnion is tapped over the forelimb region (pressure transmitted to embryo?), when the limb itself is tapped lightly, or when it is flipped by means of a needle used to pierce the amnion. It seems to make no difference whether the anterior or posterior part of the limb is tapped, the reaction is the same—a quick abduction combined with a backward movement of the forelimb. This is a local movement in the sense that it is confined to the limb and does not involve any trunk component.

The earliest record we have of a tactile stimulus eliciting a local forelimb reflex is that of a 15 mm. specimen. Data are meager, however; and it would not be surprising to find responses before this. Sometimes faradic shock was followed by similar movements in embryos at the beginning of the twenty-fifth day (13.5–14.0 mm.). One could be reasonably certain of their presence only when the reflexes came after the initial movements caused by direct muscle stimulation and when they occurred in the opposite direction (reflex backward following initial myogenic forward movement; 14–15 mm.) or when stimulation of the tips of the digits (volar aspect) resulted in movements at proximal joints. The fact that pressure is an adequate stimulus before touch leads one to consider the possibility that a primitive type of proprioception is present in the cat before exteroceptive "sensation" develops.

Is there evidence that foreleg reflexes are primary in animals other than the cat? Orr and Windle (1934) found that a wing-bud twitch, similar to the foreleg reflex, can be elicited from chick embryos before any other response can be induced, and that this response is correlated with the appearance of reflex arcs in the spinal cord (Windle and Orr, 1934). In this animal, spontaneous movements of the neck and trunk, but not the wing, precede the wing reflex. Angulo (1932) did not report movements of the forelimbs in rat embryos younger than 388 hours after insemination (14.5–16.5 mm.). In the first place, it is doubtful if he applied stimuli adequate to elicit them; and, secondly, his experimental animals were maintained under ether anesthesia, which may have interfered with normal reactions. In a thesis (which unfortunately has never been published), Swenson (1926) recorded responses of the forelimb following snout stimulation and accompanying lateral head flexion as early as 379 hours. We have had occasion to examine a few rat embryos in this laboratory and have obtained results which are similar to those on the cat. Foreleg reflexes were observed somewhat earlier than by either Angulo or Swenson; in fact, between $376\frac{1}{2}$ and $378\frac{1}{2}$ hours (12.5 mm.–13.5 mm.) little motility except that of the forelimbs was obtained with mechanical stimuli (Windle and Minear, unpublished observations). It may be significant that we avoided the use of anesthesia while studying the embryos; this was made possible by previously decerebrating (ligating basilar and carotid arteries) the mother rats (Windle and Minear, 1933). The earliest movements Coronios (1933) has seen in the cat are lateral head flexion and questionable retraction of the forelegs in one litter whose average length was 19.2 mm. and whose estimated fertilization age was 23 days (copulation age, 25 days). In the human species, Yanase (1907), reported movements of the upper extremity of a 20-mm. fetus, and Strassman (1903) found slow arm and leg movements in one which measured 22 mm. after fixation. Minkowski (1923) saw movements in no specimen smaller than 30 mm. long. One cannot accept any of these results on the human without reservations, however, because physiological conditions were far from perfect. The first reflexes of the human embryo unquestionably have not been observed.

FURTHER DEVELOPMENT OF FORELEG REFLEXES

During the course of development of the cat embryo other reflexes make their appearance in the forelimb. Just as the first response at the shoulder can be elicited before the limbs move spontaneously or before they flex with the trunk movements, so local reflexes at the elbow and wrist appear as separate entities before the distal part of the limb moves in an integrated pattern initiated in the trunk. Thus, by flipping the forelimb lightly, a local flexion can sometimes be obtained at the elbow in embryos as small as 16 mm. long (25 days). True, it is often followed by other more distant movements, such as bending at the shoulder or extension of the head and flexion of the trunk; but the point we wish to stress now is that other more proximal or cephalic movements and reflexes were never followed by local elbow flexion at this stage. Similarly, local wrist movements are at first unrelated to others. The first response was thought to have been seen in one 16.5 mm. embryo, but usually the wrist cannot be made to extend or flex before the beginning of the twenty-seventh day (17.5–18.5 mm. long).

THE APPEARANCE OF NECK REFLEXES

Another primitive reflex which appears in cat embryos at about the same time, or possibly very soon after that of the foreleg, is the "snout reflex," a contraction of the lateral or dorsolateral neck musculature elicited by stimulating the nose. Here again, strong stimulation produces a response before light tactile stimuli are adequate. However, the embryo does not at first respond to pressure on the nose as readily as it does to faradic shock. This might be expected because the receptors on the snout are undoubtedly exteroceptive ones.

The smallest embryo from which we were able to elicit a reflex extension of the head by stimulating the snout with faradic current was 13 mm. long (23 days, 10 hours, copulation age). Another of the same litter 13.5 mm. long and one of unknown age 14 mm. long gave questionable reactions. Definite reflexes were obtained in the case of one 14.5 mm. litter-mate of the latter. In none of these specimens was it possible to elicit the "snout reflex" by touching or tapping on the nose, but in each of those of unknown age it was thought a slight head movement occurred spontaneously at the moment of delivery.

This movement, if such it was, may have been due to handling the embryos and might tentatively be considered a pressure response. The 13.0–14.5 mm. specimens of litter 15:33 all showed the snout reflex to faradic shocks, and one of 14 mm. also responded to tapping and possibly to touching the nose. These embryos were 25 days old, and their behavior may be advanced for their size (footnote ††, Table I).

In a previous article (Windle, O'Donnell, and Glasshagle, 1933), mention was made of very slight head movements in 13 mm. and 13.5 mm. embryos. At that time it was thought that the movements were passive and occurred on account of contraction of the shoulder muscles which likewise moved the forelimb. In the light of our recent studies it is now believed that they may have been truly active movements of the head. There is little question that, had faradic stimuli been applied to the snout instead of confining experimentation to touch and pressure, a larger number of embryos would have exhibited extension of the head. It is possible that the weight of the embryonic head is a factor that makes it difficult to elicit movement in the neck as early as the forelimb.

Although the first response to faradic shock applied to the nose is usually a quick extension of the head, touching or tapping (when these forms become adequate) often results in a lateral flexion of the head toward the side opposite that stimulated. It is believed that direct extension may result from bilateral stimulation of a large cutaneous area at the end of the snout, whereas the lateral movement follows more localized stimulation. The character of the first reflex head movements is similar to that of the early foreleg reflexes.

That movements of the head, involving contractions of the muscles in the neck or rostral part of the trunk, are among the first to appear in embryos, has been the conclusion of many investigators. It is not necessary to consider the literature on this phase in development here, but the attention of those who are interested should be directed to a recent article by Carmichael (1933), where the whole subject of development of behavior is admirably reviewed.

FURTHER DEVELOPMENT OF NECK REFLEXES

Some astonishing changes seem to take place in the head responses as development proceeds. It soon becomes evident that a brief

shock applied to the lateral side of the snout almost invariably elicits a movement of the head to the opposite side. This lateral movement accompanies head extension. It may be superimposed on the extension so that the resulting movement is at first slightly backward and then to the opposite side. Faradic stimulation of the side of the snout did not elicit this reflex in specimens much smaller than 14 mm. long, except in the case of litter 15:33, whose members may be abnormally short for their age. Stimulation of all parts of the head except the eye region (as they become reflexogenous zones) results in this same reaction in most specimens smaller than 17 mm. long. Faradic stimulation of the eye region, on the other hand, invariably results in a head extension with flexion to the same side. This was seen best in embryos 16 mm. and more long but was encountered in a few smaller specimens.

On occasion, tapping, touching, or stimulating the side of the snout of 16 mm. and 17.5 mm. embryos with faradic shocks was followed by a movement of the head to the same, instead of the opposite side. In embryos as large as 20–22 mm. long, the usual response to stimulation of the side of the nose or lower jaw was an extension of the head and a flexion to the stimulated side. However, stimulation of the ear still produced a flexion to the opposite side. It will be very interesting to try to determine whether changes take place in the central nervous system that can account for this remarkable reversal from a contralateral to a homolateral reaction pattern.

THE APPEARANCE OF HIND-LIMB AND OTHER REFLEXES

The hind limbs can be induced to move before the time they become involved in the general massive reactions of the embryo. Hind-limb reflexes recapitulate those of the forelimbs and are comparable in every way. In one 15 mm. embryo of litter 10:33, 24 days and 1 hour old, a very slight abduction (flexion?) was thought to have occurred when the amnion was tapped over the "hip" region. We found no other reflexes of the hind limb prior to the beginning of the twenty-sixth day in 16 mm. specimens, but the number of observations is not as great as it was in the case of foreleg reflexes. Movements at the knee could not be induced before the early hours of the twenty-

seventh day in specimens 17.5 mm. and 18.5 mm. long; but, here again, observations are limited in number.

Another reaction which has the characteristics of a local reflex occurs after stimulation of the genital region or the base of the tail with faradic shocks. It is a quick twitch of the lower end of the trunk or pelvis which carries both hind limbs forward. It was observed in the 17.5–18.5 mm. embryos. Sometimes the locally induced reflex of one hind limb was followed by this pelvic reflex. This same lower trunk or pelvic reflex was observed several years ago in slightly larger embryos (Windle and Griffin, 1931). It puzzled us at the time because it was obviously a local response of musculature not yet involved in the massive motility pattern and therefore did not conform to the theory of individuation from a total pattern (Coghill, 1929) which we confidently expected to support.

Likewise, the earliest independent reflex movement of the tail (lateral flexion) occurred after tapping the amnion over the hind quarters of 17.5 mm. embryos. All of these locally induced reactions differ from the responses obtained by stimulating the muscle directly, as we have already stated. Furthermore, they all make their appearance before the parts involved move spontaneously. This seems to be a general rule applicable to all musculature so far studied.

THE INTEGRATION OF REFLEXES

The question arises whether the first head reflexes are associated with movements of the forelimbs or whether the two responses are separate. It is quite clear from our observations that there is usually no relationship between the two. Faradic stimulation of the snout at first produces nothing but head extension. Pressure on the limb at first produces nothing but an outward and backward movement of the limb as a whole. A gross pressure on the embryo transmitted through the amniotic fluid may result in both reflexes simultaneously and lead one to believe that the two movements are really one. This, we believe, was a point of confusion in our earlier studies (Windle, O'Donnell, and Glasshagle, 1933). The recent experiments indicate that movements of the forelimbs do not accompany head extension in very many embryos smaller than 15 mm. long; but the reflexes have

been observed separately in limbs and neck in specimens of 13 mm. (in fact, one observation of the forelimb reflex was made on a 12 mm. embryo in earlier studies).

The conception of the local reflex as the primary behavior response is, we believe, not well supported by work of other investigators because most of them have overlooked the first foreleg (probably proprioceptive) reflex in their specimens. When attention is directed to the use of mechanical (pressure) stimulation and to the need of better physiological conditions, it is probable that the independence of the earliest responses will be found in other animals.

The primitive foreleg and head reflexes do not remain separate very long. They soon become integrated (as nearly as we can judge from our records, about the beginning or the middle of the twenty-fifth day after mating). The first indication of integration was a head movement which followed a reflex of the leg. Sometimes stimulation of the foreleg of 15.0–15.5 mm. embryos by means of pressure was followed first by the local twitch of the limb and then a slight quick extension of the head either directly backward or to one side. In the same specimens, it was not possible to obtain an active reflex movement of the forelimbs by stimulating the nose either by tapping or by faradic shock. True, at about this time or slightly later (?) the "snout reflex" involves more of the rostral trunk musculature than it did at first, and consequently the forelimbs may be carried forward together when the head extends. But this motion does not characterize the foreleg reflex, which is executed in the opposite direction; it is simply an expression of the downward growth of the massive neck-trunk movement "pattern."

Throughout further development in the period of the third week, as has been stated previously (Windle, O'Donnell, and Glasshagle, 1933), we believe that we can observe the rostral progression or conduction of movements following an initial local reflex. This does not mean there are no movements proceeding in "wavelike" descending sequence. It is an observation that a local reflex anywhere in the body seems to be followed by such a descending "wave" only after impulses have been conducted rostrad from the site of the stimulus.

It is convenient to refer to the descending sequential movements as "mass reactions." The first expression of such a reaction is the

initial head movement which follows stimulation of the snout. At the time it appears, it is, in reality, a local reflex. Gradually, it expands caudad, involving more of the neck musculature, the upper trunk muscles actively and the forelimbs passively, the forelimbs actively, the lower trunk muscles actively and hind limbs passively, the hind limbs actively, and the tail. At first, such a mass movement can be elicited only by stimulation of the nose, then by stimulation of the forelimbs, then the hind limbs and genital region. Sometimes, other more rostral local movements follow the initial local reflex and precede the mass movement. We have consistent evidence that local reflexes make their appearance at any location before the musculature which they use becomes incorporated in the growing pattern of mass reactions.

The development of the sequential movements was described in a previous article (Windle, O'Donnell, and Glasshagle, 1933), and the present observations can do little but add confirmation to the account found there. It is the descending sequence of mass movements that characterize the writhing, squirming fetuses of the last days in the third prenatal week, it is this "syndrome" that foreshadows the development of the postural and locomotor reactions of the newborn kitten. If one consults our earlier studies (Windle and Griffin, 1931), one realizes that it is little other than this pattern of behavior that we saw and reported at that time, because the sequential mass movements are probably the only ones that manifest themselves spontaneously and almost the only ones that appear when very mild forms of stimuli are applied to embryos and young fetuses whose cutaneous reflexogenous zones are still largely confined to the head region and are undeveloped elsewhere. This criticism of our own work may apply to that of other investigators (Swenson, 1926; Tuge, 1931, 1934; Kuo, 1932; Angulo, 1932; Coronios, 1933).

OTHER EMBRYONIC MOVEMENTS

Certain behavioral reactions which may be similar to some of the asphyxial movements described by Angulo (1930) were studied in specimens of six litters (13:33, 18:33, 11:33, 14:33, 17:33, 16:33) of the present series. This investigator reported that 18-day rat fetuses showed no responses "to tactile stimulation of the hands. But, on ligation of the umbilical cord they give a very characteristic

waving of the hand shortly after the time of ligature." Likewise, before the hind limbs move reflexly, "on ligation of the umbilical cord the first observable movement is flexion of the hind legs which is then followed by waving of the hands and the characteristic body movements of this age." He believes his observations indicate that centers were stimulated directly by means of metabolites (CO_2) (see also Angulo, 1933*a*).

We observed vermiform, "waving" movements in sixteen cat embryos but found no constant relationship between time of ligation of umbilical cord and the appearance of the first waving of the forelimb, hind limb, or tail. In one instance, the movement occurred 22 minutes after ligation; in another, 72 minutes afterward. There does seem to be a relationship between time of delivery, time of severing the umbilical cord with bleeding, and time of appearance of the first waving movements. If a long interval has elapsed between separation of the placenta from the uterine wall and bleeding the embryo, waving movements occur promptly; thus, in one case, they appeared within 1 minute after bleeding but 74 minutes after delivery. Conversely, if a short interval has elapsed, one must wait some time for them to appear; for example, another embryo failed to exhibit waving movements for $11\frac{1}{2}$ minutes after cutting the umbilical cord, or $27\frac{1}{2}$ minutes after delivery. In three of our sixteen cases, very slight movements appeared before the embryos were bled; but the specimens were cold. The best responses came after severing the cord; in fact, some appeared after the heart had ceased to beat. We observed no movements of this kind in specimens kept warm; all occurred in embryos and fetuses allowed to cool to room temperature.

We do not profess to know what their significance is. The fact that they seem to bear a relationship to bleeding or cooling the embryos, and have in no case appeared in the normal specimens, suggests that they are death phenomena. We simply wish to call attention to them and to emphasize the point that we are not confusing true reflexes and other associated movements in the cat with such abnormal responses. As has already been stated, the studies on normal behavioral reactions and reflexes were conducted on embryos in the early minutes after delivery of the specimens, while physiological conditions were at their best.

SUMMARY

Direct stimulation of muscle elicits movements in 23-day-old embryos (copulation age), which exhibit no spontaneous behavior and from which no reflexes can be obtained. These movements possess characteristics which differentiate them from the first reflexes.

The first reflexes appear early on the twenty-fourth day in some specimens. They are (1) abduction with movement of the forelimb backward and (2) extension with lateral flexion of the head. The former occur in response to pressure stimuli before they can be elicited by faradic shocks and touch. The latter occur in response to faradic shocks before they can be obtained with pressure and touch. At a later time reflexes at the elbow (25 days) and at the wrist (26 days) appear. Abduction at the hip makes its appearance in response to pressure stimuli between the twenty-fifth and twenty-sixth days, but knee movements are later (26+ days). Local flexion of the pelvis and lateral tail movements can be elicited at about this time (26+ days).

All these reactions have characteristics of local reflexes. Each seems to occur for the first time unassociated with the others, and can be elicited only by applying the adequate stimulus locally. Soon after any one reflex appears, the local movements may be followed by a more rostral response; sometimes a caudocephalic series of sequential movements is seen. The impulse which incites such a series seems to elicit other cephalocaudal sequential movements (mass reaction) upon reaching the head. However, the descending series of movements does not involve the more caudal musculature recently activated in local reflexes.

Although the first response elicited by stimulating one side of the snout is a contralateral flexion of the head superimposed, apparently, on head extension, this reaction becomes reversed during the twenty-sixth day. By the twenty-eighth day, stimulation of the side of the snout or jaw causes a movement of the head to the same side. The first response obtained from the eye region is a homolateral head flexion, and this does not become reversed during the third fetal week.

Certain vermiform movements of the extremities are observed in dying embryos which have been allowed to bleed and cool. Their

significance is not known. They were not confused with other reactions described above because the experiments on reflexes were confined to the brief period after delivery when physiological conditions were as nearly as possible perfect.

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THE RÔLE OF BACTERIA IN THE GROWTH OF COLPIDIUM COLPODA[1]

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RECENT years have seen the introduction of methods of control in the culture of protozoa which supplant the old infusions with their totally unknown bacterial flora. This advance is particularly needed in the case of this phylum because the "internal environment" described by Claude Bernard, which insures at least a relative constancy for the higher organisms, is lacking in the single-celled animals.

Two types of culture method have developed more or less simultaneously: first, in the absence of other life, i.e., pure culture; second, on known strains of bacteria, or other suitable food organisms.

Oehler (1919) was the first to achieve pure culture in a ciliate. Isolating individuals of *Colpoda steinii* which had migrated over the surface of an agar plate he was able to obtain sterile organisms, and to grow them in 2 per cent peptone-glucose and dead *Bact. coli*, or finely divided egg albumen, but not in particle-free media.

Similar pure cultures were obtained by Oehler (1924) with *Colpoda cucullus;* by Lwoff (1924) (also later by Chatton and by Milkovitch) with *Glaucoma piriformis;* by Peters (1929) with *Colpidium colpoda;*[3] by Butterfield (1929) with "*Colpidium*";[4] by Hetherington (1933) with *Colpidium campylum*, *Glaucoma scintillans*, and *Loxocephalus granulosus;* and by Elliott (1933) with "*Colpidium stria-*

[1] The author is indebted to Dr. M. B. Harris for identification of the bacteria, to Dr. L. Michaelis for suggestions concerning methods for establishing reduction potentials, and to Dr. C. B. Van Niel and Dr. S. O. Mast for critical reading of the manuscript.

[2] Adam T. Bruce Fellow, 1932–33.

[3] The identification of this ciliate appears to the author uncertain.

[4] This ciliate, cultures of which Dr. Butterfield very kindly sent me, is *Glaucoma piriformis*. This identification was verified by Dr. André Lwoff, using his precise silver method.

tum."[5] Glaser and Coria (1933) have also reported the pure culture of *Paramecium caudatum*, but it is important to note the very deceiving behavior of certain slow-growing, adherent, fresh water bacteria described in my paper (1933, p. 260). Since they resemble the "patches of cilia" observed by Glaser, the purity of his cultures is open to question.

The second method, that of culture on known strains of bacteria, developed particularly with reference to *Paramecium*, for this ciliate has resisted all efforts, at least up to the present year, of the numerous investigators who have tried to establish pure culture. Beginning with the work of Hargitt and Fray (1917) and of Purdy and Butterfield (1918), the technique has perhaps been most refined in the hands of Raffel (1930).

Peters (1921) maintained that *Colpidium colpoda* can grow in pure culture on inorganic sources of nitrogen such as ammonium glycerophosphate. However, his cultures actually contained bacteria (Gatenby and Cowdry, 1928, p. 412), and his organism is more recently (Peters, 1929) reported in pure culture on a medium of yeast extract containing suspended particles. Bresslau (1923) distinguished in a very clear manner between the large and small strains of "*Colpidium colpoda*," naming the latter *Colpidium campylum*. The mean length of Peters' ciliate, given (1921) as 50 μ, thus identifies it as *C. campylum*, for *C. colpoda* averages 110 μ in length.

A study of the cultural requirements of *C. colpoda* is interesting because (1) it is closely related to, yet twice as large as, any of the other ciliates which have been obtained in pure culture, (2) some light may be thrown upon the nature of these requirements in forms which are at present much more difficult to grow.

The growth of *C. colpoda* is studied in the present paper first with reference to living bacteria both in non-nutrient salt solutions and in solutions of organic compounds of varying complexity; second, in pure culture under a variety of chemical and physical conditions.

METHOD

Temperature.—The temperature for the experiments to be reported varied between 23° and 25° C.

[5] This ciliate, cultures of which Dr. Elliott very kindly sent me, closely resembles *Glaucoma piriformis*.

Medium.—The inorganic medium, Peters' medium, to which nutritive substances may be added, has been given (Hetherington, 1933). Calcium hydroxide is brought into solution with excess CO_2 [forming the $Ca(HCO_3)_2$], then the other constituents are added. Experience shows that excess CO_2, rather than the measured amount, facilitates preparation, preserves the medium from the growth of small monads and bacteria, and prevents precipitation when autoclaved. The following modification is simpler to prepare, but is preferable only for the preparation of complex nutrient media because phosphate is lacking and the hydrogen ion concentration is not as stable:

$CaCl_2$	0.00055 M.
$MgSO_4$	0.00015 M.
K_2CO_3	0.00015 M.
Na_2CO_3	0.000075 M.

The theory of this cation balance is reported elsewhere (Hetherington, 1932*c*). Since this paper, Penn (1933) has tested an extensive series of physiological media for *Pleurotricha*, and has found Peters' medium the best.

It is essential to use chemicals which do not contain silver. The sodium in the above system was introduced in the form of a Kahlbaum preparation of sodium silicate because of its beneficial effect as reported by Pace (1933). This proved entirely unsatisfactory: a spectroscopic analysis revealed traces of silver (cf. Ball, 1933).

Many of the experiments reported here have been repeated using an alkaline spring water ("Chattolanee"—Baltimore), whose analysis reveals a resemblance to Peters' medium, several on an acid spring water, analysis unknown. The former was equal to or slightly less favorable than Peters' medium, the latter unsatisfactory.

Difco peptone was used. Concentrations of peptone-glucose are totals, the value for each constituent being one-half this amount. The yeast extract used does not correspond to the extract of autolyzed yeast cells referred to in the previous paper. It is prepared according to Ayres and Rupp (1920). The hydrogen ion concentration will be correct if it is autoclaved in the presence of excess $CaCO_3$.

Fresh lettuce (5 per cent), and wheat grains (2 per cent), when

autoclaved in the presence of excess $CaCO_3$, have been found to support excellent cultures of *Colpidium*.

Glassware.—Unless specified, 125 cc. pyrex Erlenmeyer flasks were used for culture in nutrient media. Tests of growth on bacteria in salt solutions (Peters' medium) were carried out in Columbia isolation dishes.[6] They may be placed in petri dishes, which are then wrapped in paper for sterilization in hot air (170° C. for one hour).

Calculation of numbers and division-rates.—Estimation of the number of Colpidia in nutritive media was made by counting diluted samples as follows: from 0.5 to 2 cc., depending upon the approximate concentration of the ciliates, is removed from the stirred culture and diluted with water to 20 cc. After careful stirring at least eight volumes of 0.05 cc. each are sampled. These are counted directly under the low power of the binocular. The following formula gives the number of Colpidia per cc. in the original culture:

$$\frac{X}{N}=\frac{D}{V} \quad \text{or} \quad X=\frac{DN}{V},$$

where X = the number or organisms per cc. in original culture,
D = number of times original sample is diluted,
V = volume of final sample.
N = average number organisms in final sample.

The average number of divisions (i.e., generations) per organism in a culture where the initial and final numbers of individuals are known is given by the following formula:

$$\frac{\log \text{ final number} - \log \text{ initial number}}{\log 2}.$$

The division rate, the number of divisions per organism per day, is obtained by dividing the above result by the age of the culture in days.

Bacteriological technic and precautions.—Bacteriological methods have been rigidly adhered to in the manipulation of cultures reported in this paper. Special "stock" flasks, from which only the definitive transfer to new stock flasks is made, have been maintained. Cello-

[6] Watch glasses No. 9851, Arthur H. Thomas Co., Philadelphia.

phane over the cotton and tied around the neck of the flask prevents the accumulation of dust at the lip. The definitive transfer (subculture) has been carried out when there was minimum disturbance of the air. Parallel stock flasks are carried, from which the transfers to experimental vessels are made. Nutritive agar plates were used freely for testing the bacterial purity of the cultures.

Some of the results reported, especially those involving changes in behavior of the ciliates after months of subculture, have occasioned experimental verification of a rather arduous nature. Glassware, chemicals, water, light, temperature, and bacteria must be under the control of the investigator, and conditions must be subject to duplication over long periods of time. Only then may inherent characteristics of *Colpidium* be demonstrated. The experiments reported in the following pages have been controlled as carefully as possible and repeated. Others were discarded because of the probability of unknown factors.

The technique of using pure cultures of two kinds of organisms in the same culture vessel has been termed "*zweigliedrige Kultur*."

GROWTH ON AEROBACTER AEROGENES

My previous studies (1933) on bacteria obtained from the bacteriology department of Stanford University revealed *Aerobacter aerogenes* as one of the most suitable for the growth of *Colpidium*. Quantitative studies on this bacterium are presented here to permit comparison with the bacteria isolated from natural waters.

Concentration of medium.—Yeast extract is less satisfactory than peptone and glucose. Experiments wherein the total concentration and the proportions of the latter were varied showed that equal amounts give best growth at a concentration of 0.2 per cent and that reduction in the concentration of peptone is better tolerated than disproportion in the opposite direction. Below 0.2 per cent the total yield drops off rapidly; above, an increasing fraction of the ciliates are found dead, apparently due to acid formation.

At a later time the concentration series was repeated in culture tubes containing 10 cc. of medium. No injury, using an inoculum of 0.2 cc., was observed in concentrations up to 3.5 per cent. The latter produced 11,300 ciliates per cc. on the fifth day (pH 6.8). The 5 per cent concentration gave a small growth after two weeks.

Isolation of one animal.—If one individual of *C. colpoda* is sterilized and placed in 50 cc. of 0.2 per cent peptone glucose simultaneously inoculated with a small amount of *Aerobacter aerogenes*, the system will become cloudy from bacterial growth in less than 24 hours. On the fourth day numbers of *Colpidium* will be visible upon inspection with the unaided eye. Three days thereafter the cloudiness due to the bacteria will disappear, leaving a clear system supporting large numbers of sharply defined bodies, which swim about actively. Upon microscopic examination the Colpidia will be seen to have many food vacuoles (12–23). The number of organisms will be 850–1,250 per cc. on the sixth day, 5,000–5,500 on the seventh, and 8,550–9,500 on the eighth. The seventh-day count represents a division rate of 2.58. Increase is small after the eighth day.

If a large amount of *A. aerogenes* (50–100 mg.) scraped from an agar plate is added, early growth of the Colpidia will be more rapid, but the final number will be slightly lower. Numbers will be visible on the third day, and on the sixth day there will be approximately 7,750 (=division rate of 3.09), on the seventh, 7,980, and on the eighth, 8,100.

If 0.5 cc. of such a culture is introduced into a similar nutrient medium it will again cloud, but after only 36 hours large numbers of Colpidia will be appreciable, and within 48 hours the cloudiness will have disappeared. This subculture will show a concentration of approximately 10,000 per cc. on the fourth day. Increase will be small after the fourth day.

The consequences of varying the volume of the inoculum between 0.05 cc. and 28 cc. is given in Table I. The inoculating medium was a growing culture supporting about 6,000 per cc. An inoculum of 0.1 cc. containing about 600 Colpidia results after 5 days in three times as many organisms (12,200 per cc.) as an inoculum of 3.2 cc. containing 19,000 Colpidia. They were evenly suspended in the 0.05 cc.–0.8 cc. flasks, but aggregated downward in the others.

The consequences of varying the relative surface of the culture exposed to air are given in Table II. A culture having a small ratio of surface to volume, 0.16, gives 2.5 times as many organisms as one whose ratio is more than 6 times as large, 1.04. The 0.10 ratio supported animals of excellent appearance but which had not increased

as rapidly as the others. Under the microscope the ciliates in the 1.54–0.55 flasks appeared thin, with only 2–6 food vacuoles.

TABLE I

THE EFFECT OF AMOUNT OF INOCULUM OF *Colpidium* GROWING ON *Aerobacter aerogenes*

Final volume = 60 cc. Counts made on fifth day.

Volume of Inoculum (Cc.)	Number of Colpidia Per Cc.
0.05	7,310
0.1	12,200
0.2	10,500
0.4	11,200
0.8	9,500
1.6	4,950
3.2	4,000
6.4	4,350
10.0	4,600
12.8	5,110
15.0	5,960
20.0	8,000
28.0	7,520

TABLE II

THE EFFECT OF VARYING SURFACE/VOLUME ON A CULTURE OF *Colpidium* GROWING ON *Aerobacter aerogenes*

Inoculum 0.5 cc.; counts made on fifth day.

Volume (Cc.)	Diameter of Surface (Cm.)	Surface/Volume	Number of Colpidia Per Cc.
20	6.26	30.8/20 = 1.54	3,900
100*	11.5	104 /100 = 1.04	4,400
35	6.24	30.6/35 = 0.88	4,000
50	5.89	27.3/50 = 0.55	5,120
60	5.26	21.6/60 = 0.36	5,400
80	5.12	20.6/80 = 0.26	9,300
100	4.44	15.5/100 = 0.16	11,100
115	3.84	11.6/115 = 0.10	8,200

* Prepared in 1 l. flask; all others in 125 cc. flasks.

Infection.—After the first three months of culture, subcultures altered in behavior; the medium became strongly acid and remained cloudy. No foreign colonies appeared on nutritive agar plates, and

examination revealed the characteristic rods of *A. aerogenes*. It was thought at first that the bacteria had undergone change after the fashion of Arkwright's "R" variant (1921), but since results could not be duplicated the difficulty was referred to infection.

The ciliates in these presumably infected cultures never reached the relatively large numbers characteristic of the previous ones. Increase was slow; the average of a number of counts gave 680 per cc. on the fifth day and 2,000 on the tenth. The rate of increase is enhanced if the medium is prepared in the presence of $CaCO_3$. Decreasing the surface exposed to air in the culture vessel increases the yield and results in delayed clarification. If one animal was subcultured the division rate was about 1.16 for 12 days. After 7 days there were 8 per cc., and as follows on ensuing days: eighth, 16; ninth, 35; tenth, 108; eleventh, 236; twelfth, 525. After the twelfth day more or less evidence of injury (vacuolization) is apparent. A *Colpidium* was removed from such a culture, washed through suspensions of fresh *A. aerogenes* until the original bacteria were displaced. This culture subcultured with remarkable uniformity giving 10–11,000 per cc. every 5 days for the 22 months during which it was maintained.

GROWTH ON BACTERIA ISOLATED FROM NATURAL WATERS

Pure cultures of nine species of free-living bacteria derived from fresh water ponds were prepared. The identification, following Bergey (1930), follows.

1. *Escherichia communior* (encapsulated). Encapsulated forms are common in strains of animal origin. Isolated from an old wheat infusion supporting large numbers of *Stentor coeruleus*. The infusion had cleared completely.

2. *Achromobacter inunctum*. Isolated from an earlier stage of a similar wheat infusion supporting a rapidly increasing population of *Stentor*.

3. *Achromobacter rodonatum*. Isolated from a vessel of decaying filaments of algae supporting large numbers of *Urocentrum turbo*. The great majority of the bacteria appeared to be this form.

4. *Micrococcus conglomeratus*. A few ochre colored colonies appeared on plates from the above (3) culture, pure cultures of which were obtained.

5. *Aerobacter aerogenes*. Isolated from the surface of a hay infusion supporting a number of protozoan forms including *Stentor coeruleus*.

6. *Achromobacter?* Isolated from below the surface of above infusion (5), a region which was quite clear and supported *Colpidium colpoda*. Tests follow.

Small slender gram-negative rod, non-motile (?).

Gelatin	negative	Cellulose	negative
Agar colonies very slow growth, spreading.		Lactose	slightly alkaline
		Dextrose	slightly alkaline
Milk	no change	Saccharose	slightly alkaline
Potato	no growth	Raffinose	slightly alkaline
Indol	negative	Maltose	slightly alkaline
Nitrate	negative	Salicin	slightly alkaline
Starch	negative	Citrate	negative
H_2S	negative		

7. *Micrococcus*. Unfortunately these bacteria died on the nutritive agar surface before the tests were run. Possibly an accidental exposure to sunlight was responsible. Isolated from the excellent cultures of *Bursaria truncatella* maintained for a number of months by Penn (1932). They were morphologically the only distinctive bacteria, and were not predominant numerically.

8. *Achromobacter?* Isolated from a hay infusion which, instead of undergoing the usual clouding and acid bacterial growth, cleared in two days. Tests were identical with 6.

9. *Bacillus niger*. Isolated from an old and excellent rice culture of *Amoeba proteus*. The plates revealed no other kinds of bacteria.

GROWTH ON BACTERIA IN NON-NUTRIENT SALT SOLUTION (PETERS' MEDIUM)

The method for testing growth of *Colpidium* on bacteria suspended in Peters' medium previously described (Hetherington, 1933) has been modified. The bacteria removed from the 24-hour plate are pushed off en masse with the aid of another needle. The mass will disintegrate to some extent but a gradient of concentrations of bacteria is created of which the optimum may be selected by the protozoa. Four Colpidia are introduced into each 1 cc. volume of freshly prepared medium each day. The previous technique has

also been modified with reference to treatment of dishes. They were simply cleaned in the former experiments, for it was assumed that any infection would be negligible in the absence of nutrient. This assumption is not justified, and all dishes and petri dishes were dry-air sterilized for the following experiments.

Table III summarizes results for each of the nine bacteria, and, for comparison, on *A. aerogenes*. The figures at the base of each column represent the average division rate. Three columns appear under the heading of *A. aerogenes*. The first gives counts on bacteria which had been previously heated to 60° C. for 5 minutes, the second is the unmodified experiment, the third consists of bacteria in 0.2 per cent peptone-glucose instead of Peters' medium. Two additional experiments suggest themselves, each of which has been performed. The first, growth on suspensions of washed bacteria, has been reported (Hetherington, 1933, p. 269). The second, growth on suspensions of dead bacteria in complex nutrient, will be reported as a part of extensive efforts to achieve pure culture.

The division rate on *A. aerogenes*+*Escherichia communior* is intermediate between that on the two separately. As in the case of other mixtures tried, no acceleration in growth is obtained.

Two sets of results appear under *Achromobacter rodonatum* and under *Aerobacter aerogenes* 5. The first, Column A, gives the result of the first trial. The second, Column B, is a repetition. Another repetition gave results resembling Column B. Speaking in general, however, mere repetition of these experiments gave somewhat more uniform results.

The relatively high division rate of 3.17 on *Achromobacter* 6 may be correlated with their great activity (i.e., metabolism). Instead of remaining in the center of the dish as a rather dense mass, considerable dissolution occurred, and a large ring of bacteria formed overnight at the periphery of the medium.

Since four colpidia were introduced into fresh dishes daily, increase on *Micrococcus* 7 is seen to be nil. No ingestion of bacteria whatever was observed.

At the end of the fourth day of transfer on *Achromobacter* 8 the animals were reduced in size, with an average length of 63 μ. The few food vacuoles present were oriented posteriorly.

Why should *Colpidium colpoda* grow so erratically on bacteria in salt solution? In a previous paper (1933) the author tried washing the *Aerobacter aerogenes* before feeding, with the idea that toxic

TABLE III

GROWTH OF *Colpidium* ON BACTERIA IN PETERS' MEDIUM

Four *Colpidia* transferred daily: numbers give daily counts.

Days	*Aerobacter aerogenes*			*Escherichia communior*	*Aerobacter aerogenes* + *Escherichia communior*	*Achromobacter inunctum*	*Achromobacter rodonatum*	
	Dead Susp.	Live Susp.	Nutrient Susp.				A	B
1	4	38	70	8	42	15	16	8
2	4	33	84	22	29	20	8	24
3	disc.	20	77	30	16	25	2	13
4	.....	32	69	25	17	19	.....	18
5	.....	8	91	28	32	20	.....	32
6	.....	25	86	36	22	30	.....	16
7	.....	32	86	22	26	16	.....	18
Average division rate		2.74	4.33	2.61	2.72	2.37	.....	2.20

Days	*Micrococcus conglomeratus*	*Aerobacter aerogenes*		6 *Achromobacter?*	7 *Micrococcus*	8 *Achromobacter?*	*Bacillus niger*
		A	B				
1	7	8	8	16	4	17	24
2	14	8	23	56	4	19	18
3	21	2	32	24	disc.	6	25
4	19	.....	45	54	.....	4	46
5	15	.....	32	35	.....	.....	44
6	17	.....	16	20	.....	.....	8
7	18	.....	20	47	.....	.....	16
Average division rate 1.99		.....	2.65	3.17	.....	.....	2.70

metabolites carried over from the plate might be removed. No benefit was observed, however. In washing, it was found impossible to completely displace the bacteria to the end of the centrifuge tube, leaving a clear supernatant fluid. Possibly those bacteria which did centrifuge were actually aggregates rather than washed individuals.

Bacillus niger (9) is a somewhat larger bacterium which, 24 hours after streaking on a nutritive agar plate, adheres with remarkable tenacity, but 24 hours later is easily removed and suspended in Peters' medium with very few, if any, clumps. This organism centrifuges somewhat more readily than *A. aerogenes;* probably also the individuals are more effectively washed. *B. niger* was washed twice in 15 cc. centrifuge tubes, then introduced into the dishes as in the previous experiments. Colpidia removed from dishes of unwashed *B. niger* divide a few times, irregularly, when placed on the washed bacteria, then starve. They may swim about slowly for as many as 5 days before death.

In general, *C. colpoda* reacts to bacteria in Peters' medium by ingesting only a very few out of the great number which are swept by the mouth. When the bacteria are washed, ingestion is still more curtailed. This contrasts with the situation in the smaller forms, for instance *C. campylum*, where the suspended bacteria appear to be completely removed from the medium.

The bacteria removed from an agar plate by means of a platinum needle undoubtedly retain not only small amounts of metabolites but also some of the nutritive substances. This should provide for the continuance of more or less metabolism in Peters' medium.

In this connection an instructive series may be indicated: *Bacillus niger* washed, supports a few divisions only. Unwashed, the bacteria give no visible evidence of metabolism, but support irregular growth of *Colpidium* with a mean division rate of 2.70. The only bacterium clearly exhibiting metabolism following introduction into Peters' medium, *Achrombacter* 6, supported much the highest division rate of any tried, 3.17.

These results suggest that the consequences of the metabolism of bacteria are crucial for the growth of *Colpidium colpoda*. This would explain the erratic growth observed, for the amount of nutritive substances carried over with the bacteria on succeeding days doubtless varies greatly.

It is interesting that ciliates may die out even after a long period of growth, when cultured on bacteria in non-nutrient media. Thus, *Euplotes Taylori* ceased dividing after 3 years of culture in artificial sea water plus bacteria (Luck, Sheets, Thomas, 1931).

GROWTH IN NUTRIENT SOLUTIONS

Enough Colpidia were sterilized for inoculation (1 each) into the following concentrations of peptone-glucose: 0.2, 0.1, 0.05, 0.025, 0.01 per cent, and the following concentrations of yeast extract: 0.1, 0.05, 0.025, 0.01 and 0.005 per cent. Also direct transfers from cultures on *A. aerogenes* into the cultures of new bacteria were made. Apparently the former was displaced in some cases, but *Micrococcus conglomeratus* and *Bacillus niger* clearly failed to overgrow and displace the original *A. aerogenes*.

1. ESCHERICHIA COMMUNIOR

A successful culture was obtained from an inoculum of one sterile[7] *Colpidium* in peptone-glucose. The first two subcultures remained cloudy, giving only 1,000–2,000 per cc. The third subculture cleared in 4 days (inoculum, 0.5 cc.), giving 9,750 per cc. on the fifth day. This has continued without change for 21 months. A series of counts gave 2,060 per cc. on the third day and 5,800, 9,900, and 11,000 on the fourth to sixth days. Decreasing the surface has little effect on the rate of increase. However, the ciliates are smaller in the shallow volumes, averaging 80–110 μ against 90 120 μ for the deep. Also, the former appear to be cleared somewhat less promptly. Increasing the volume of the inoculum increases the final yield to some extent, contrary to the case of *A. aerogenes*.

Growth on this encapsulated bacterium demonstrates the absence of any crucial need of flagella for normal ingestion of food in the case of *Colpidium colpoda*.

2. ACHROMOBACTER INUNCTUM

The division rate in Peters' medium, 2.37, is somewhat lower than for *Aerobacter* and *Escherichia*. In nutrient media, 5,400 is the maximum which was obtained. An isolated ciliate gave 4,300 per cc. in 10 days on peptone-glucose. Subcultures continued without apparent variation for 8 months. Clarification is quite complete, leaving a crystal-clear fluid in which well-nourished ciliates are sus-

[7] They may be washed through nutrient media containing the particular bacterium. Probably it is easier to displace other bacteria using this method than it is to wash protozoa free of all bacteria, but the apparent early purity must be rigidly checked (plate out on agar) in the subsequent sub-cultures. The general method of sterilization has been presented (1933).

pended. Its initiation is a function of the size of the inoculum: 10 cc. will clear in 1 day, 2 in 2 days, 0.5 in 3, and 0.1 in 4. The yield is a function of the concentration of nutrient, 0.2 per cent averaging 4,700 per cc., 0.02 per cent, 537.

3. ACHROMOBACTER RODONATUM

The first attempt to obtain continued growth in Peters' medium failed (note Column A, Table III). Similarly, the isolated ciliate in peptone-glucose and yeast extract failed to survive. A second

TABLE IV

GROWTH OF *C. colpoda* IN DIFFERENT VOLUMES

(For surface/volume ratio see Table II.) Counts, giving organisms per cc., taken after 3 and again after 5 days. Column A = inoculum contained 450 per cc.; B = 3,130 per cc. Brackets indicate that the culture is still cloudy.

Inoculum (Cc.)	Interval (Days)	Volume					
		25 Cc.		50 Cc.		100 Cc.	
		A	B	A	B	A	B
0.1.....	3	[460]	[660]	[120]	[246]	[54]	[157]
	5	3,700	[2,940]	[2,700]	[2,380]	[1,100]	[2,880]
0.4.....	3	[520]	[900]	[310]	[577]	[255]	[587]
	5	3,700	3,060	3,300	3,000	3,350	3,560
1.....	3	[1,550]	3,070	[1,000]	[835]	[440]	[466]
	5	3,900	4,000	2,600	2,620	4,400	2,060
5.....	3	4,000	4,150	2,250	2,940	2,350	2,340
	5	3,600	4,620	3,250	3,800	3,440	3,000

trial using seven sterile organisms on 0.1 per cent yeast extract gave a small growth. Subcultures gave good growth, 3,000–5,000 per cc. Averaging about 4,500 per cc., they continued rather uniformly for 9 months. Clarification in consequence of ingestion by the ciliates is more prompt than in corresponding systems of *A. aerogenes*. Before discarding, a fresh culture was established from recently collected specimens of *C. colpoda* for comparison with the old. Averages of fourth-day counts for the old gave 3,380 per cc.; for the new, 3,460.

Table IV summarizes counts taken after 3 days and again after

5 days in different volumes and from varying inocula. Column A is a series in which the inoculum contained 450 Colpidia per cc., while the series in Column B contained 3,130 per cc. The striking characteristic of these results is the approach to an equilibrium of from 3,000 to 4,000 per cc., regardless of volume or size of inoculum.

Division rate for the inoculum containing a small number of Colpidia, Column A, is larger than that for the large, Column B. Average for A, 2.2 (1.6–2.7); B, 1.5 (1.0–2.0).

Division rate in the small inoculum (0.1 cc.) is larger than for the large (1 cc.).

Average, 0.1 cc. { A, 2.4 (2.2–2.7)
B, 1.9 (1.6–2.0)

Average, 1 cc. { A, 1.9 (1.6–2.2)
B, 1.2 (1.0–1.5)

Division rate in the small volume (25 cc.) is slower than that in the large (100 cc.), similarly inoculated. Average, small, 1.9; large, 2.2.

Division rate is fast in the case of the small volumes (25 cc.) for the first 3 days, then much slower for the last 2 days. It is faster for the first 3 days than the large volumes (100 cc.), and slower for the last 2, whether similarly or proportionately inoculated. Average for similarly inoculated:

(25 cc.), 2.1 for first 3 days; last 2 days, 0.8;
(100 cc.), 1.0 for first 3 days; last 2 days, 1.8.

Proportionately inoculated (0.1 cc. inoculum for 0.25 cc. volume and 0.4 cc. for 100 cc. volume):

(25 cc.), 2.3 for first 3 days; last 2 days, 1.3;
(100 cc.), 1.6 for first 3 days; last 2 days, 1.6.

With reference to the surface/volume relation, it is to be noted that at the time of final counting, 5 days, the ciliates in the shallow volumes were starving, while those in the 100 cc. volumes were well nourished and still dividing.

4. MICROCOCCUS CONGLOMERATUS

The most conspicuous characteristic of growth on this gram-positive chromogenic (ochre) bacterium is the large size attained by the Colpidia. While the division rate is relatively slow in Peters' medium

(only 2 per day), the individuals average 145 μ long by 80 μ wide, and show many food vacuoles which appear to be full of bacteria.

Culture was inaugurated in the series of concentrations on both peptone-glucose and yeast extract in two groups, one of which was placed in the dark room. The absence of light did not alter the result, which is as follows: The cultures cloud from bacterial growth, and *Colpidium* increases; the individuals appeared large and very well nourished. The system (0.2 per cent peptone-glucose) clears completely on the fourth day, at which time not more than 50 animals per cc. may be counted, but these individuals are conspicuous, due to their large size. Thereafter they die without disintegration and in the plump condition described. They do not all die in 1 day, but extinction is apparently complete within a week.

The clarification is doubtless due in part to ingestion, but agglutination on the part of the bacteria plays a rôle, and perhaps also autolysis.

5. AEROBACTER AEROGENES

The initial attempt to maintain *Colpidium* in Peters' medium failed (see Table III). Repetitions were successful, giving a rate of division similar to that on the Stanford strain.

This bacterium tends to agglutinate and subside in nutrient media to a much greater extent than the Stanford strain, reported upon in the first part of this paper. All initial isolations in nutrient media failed. Ten sterile Colpidia introduced into 10 cc. of 0.1 per cent yeast extract gave a small increase in spite of extensive agglutination. The following figures represent per cc. counts for the first, second, and third subcultures on 0.2 per cent peptone-glucose, and corresponding values for a new culture on the Stanford strain run simultaneously as a control. First, 4,020–5,360; second, 5,760–6,470; third, 6,100–6,150. Culture was discontinued after the sixth transfer. When compared with the initial culture or with *Colpidium*-free cultures, agglutination was definitely less intense in the sub-cultures.

6. (NOT IDENTIFIED)

While 4,200 colpidia per cc. were obtained in 4 days on 0.1 per cent yeast extract, and 3,650 on 0.1 per cent peptone-glucose, the ciliates appeared injured (vacuolated). The culture was not continued.

Perhaps this bacterium is adjusted to extremely dilute nutrient, for in the case of these bacteria in Peters' medium a conspicuous ring formed around its periphery, indicating active movement and metabolism (for 24 hours), even in the absence of all but traces of organic substances.

7. MICROCOCCUS

Behavior on this *Micrococcus* resembled that reported in the previous paper for *M. ureae*. No ingestion in Peters' medium, no growth in nutrient media, and no apparent toxic effect.

8. (NOT IDENTIFIED)

Colpidia on this bacterium in Peters' medium not only ceased dividing but were reduced in size (average length = 63 μ) on the third day. The few food vacuoles present were oriented posteriorly. Nutrient media failed to give growth.

9. BACILLUS NIGER

This gram-positive bacterium agglutinates and subsides completely within 24 hours. The large, coarse masses occupy a considerable volume in the bottom of the flask. The ciliates did not increase. Extensive efforts to obtain growth in nutrient media failed. The addition of extraneous bacilli from a 48-hour plate to create a suspension gave growth up to 3,000 per cc., but repetitions and subcultures did not behave uniformly. When contaminated with relatively small numbers of *A. aerogenes* excellent growth was obtained, even in 2.5 per cent peptone-glucose. In the presence of suspended bacilli the contamination did not increase the yield.

Efforts to prevent agglutination assuming it is acid agglutination as described by de Kruif and Northrop (1923) failed.

It is interesting to consider the growth of *Colpidium* on a given species of bacterium in varying media. We can in general predict from the division rates given in Table III the growth which will occur on peptone-glucose or on yeast extract. In the case of *Bacillus niger*, however, the phenomenon of agglutination enters in to preclude growth in nutrient media. Some discrepancy is apparent in two other cases. *Micrococcus conglomeratus* supported a division rate of 2 in Peters' medium, yielding large organisms, while death of similarly large animals ensued in peptone-glucose. Achromobacter 6

gave the highest division rate of any bacterium in Peters' medium, and caused very rapid increase in nutritive media, but in the latter marked injury was also apparent.

MIXTURES OF PURE STRAINS OF BACTERIA

A large number of cases in which two pure cultures of bacteria have grown in nutrient media in the presence of *Colpidium* were observed. When both were known to be favorable, the ciliate usually grew less well than on either alone; occasionally growth approximated that of the less favorable species. When one species was unfavorable, results were much less satisfactory. In one combination, however, that of *Micrococcus conglomeratus* with *Escherichia communior*, increase after 8 days reached a value equal to pure culture on the latter (13,000 per cc.). Apparently the *Micrococcus* inhibited the extremely vigorous ingestion of *Escherichia*, thereby allowing the latter to utilize available nutrient more completely, this being finally reflected in a larger yield of *Colpidium* protoplasm.

The general result stated above was predicted by a thoughtful protozoölogist in the face of experimental evidence then available (Cleveland, 1928, p. 277).

GROWTH ON BACTERIA IN SIMPLER NUTRIENTS

The following systems have been tried for the growth of *Colpidium:*

1. *Micrococcus ureae* on urea+dextrose.
2. *Pseudomonas fluorescens* on asparagin.
3. *Aerobacter aerogenes* on uric acid+glycerol.
4. *Proteus vulgaris* on glycine+dextrose.

No growth was obtained on the *Micrococcus*, and that which occurred on *Pseudomonas* could not be maintained. Good growth, after a lapse of 8–14 days, was obtained on both *Aerobacter* and *Proteus*, but the ciliates did not respond in subcultures. Possibly they starve to death before the bacteria increase sufficiently. It was found that fairly uniform growth could be maintained through at least four transfers if the bacteria were inoculated a week before introduction of the animals. This contrasts with the situation on the complex media, where, even though quite clear, *Colpidium* may appear well nourished and vigorous.

PURE CULTURE

In spite of the extensive series of failures reported in my last paper (1933), efforts to achieve pure culture have been continued. Three approaches were used. Will *Colpidium* grow in complex: (1) nutrient plus particles in suspension? (2) nutrient plus dead bacteria in suspension? (3) nutrient plus each of the above under conditions of low oxygen tension and of high reduction potential?

Particles were obtained in nutrient solutions by autoclaving yeast cells and wheat. Colpidia do not ingest the particles but swim about slowly, without dividing, for several days.

Aerobacter aerogenes was killed by heating at 90° C. for 3 minutes in 1–5 per cent peptone-glucose. The first experiments involved systems in which the bacteria were not allowed to grow previous to killing. Later, different sizes of inocula were allowed to grow for different periods, from 2 to 24 hours. As many as fifteen sterile colpidia were introduced into individual tubes; no growth was appreciated in any case.

Some of the above trials were carried out in narrow and deep tubes, so that the oxygen tension varied from surface equilibrium with air to very low values below. Tubes containing reducing sugars were boiled in alkaline solution, then neutralized, thereby generating intense reduction potentials (Eh −.10 to −.23: see Michaelis, 1933), which in some cases were maintained for several days. Similar potentials were found to exist in the sterile tubes in which bacteria had been allowed to metabolize previously for 24 hours (compare Hewitt, 1933). Failure resulted in all cases. The Colpidia swim about quite slowly, ingesting nothing, quite in contrast to their behavior in similar media containing growing bacteria. The chemical consequences of bacterial metabolism, referred to as crucial in the section on bacteria in Peters' medium, must be unstable, judging from the failure on bacteria which were killed only after considerable growth.

DISCUSSION

It is an interesting fact that *C. colpoda* is not very particular with regard to the species of bacterium upon which it will grow, although it may be more so than *C. campylum*. Indeed, the particular strain of the species used appears to be quite as important as the nature of

the larger group. Thus the coli strain from Stanford (*Escherichia coli commune*) and *Aerobacter aerogenes* 5 gave trouble, while *Escherichia communior* 1 and *A. aerogenes* from Stanford were as satisfactory as any bacterium tried.

The fact that *Urocentrum* or *Stentor* or *Amoeba* enriched on certain strains of bacteria does not imply that they are not excellent for the culture of *Colpidium* also. The experiments reported here dictate the following rules: specific bacteria supporting excellent cultures of specific protozoa will give excellent cultures of *Colpidium* (the phenomenon of agglutination in certain media may preclude growth). Other bacteria which accompany in smaller numbers are a liability, and in pure state will not give good cultures. Bacteria supporting mixed populations of protozoa give more or less satisfactory cultures, inferior to unmixed systems. There is some evidence that mixtures of bacteria (none of which is predominant) yield less abundant and vigorous protozoa; none that they are superior. Extensive experience will be necessary to establish this point.

The nutritive requirements of *C. colpoda* may be intermediate between those forms which are easy to grow and the very fastidious ciliates. In this connection a rough correlation may be demonstrated. Oehler's *Colpoda steinii*, which will grow on dead bacteria in non-nutrient medium, is the smallest of the series set forth in the introduction to this paper, averaging 28 μ–43 μ in length. The other members of the series are about 50 μ in length, and although they will grow luxuriantly on living bacteria in non-nutrient medium, are apparently not adjusted to dead bacteria under the same conditions.

Turning to *C. colpoda*, which averages 110 μ in length, we find pure culture to be impossible, growth on living bacteria in non-nutrient solution precarious and dependent upon small amounts of substances in solution. Apparently very unstable substances.

It is not at present possible to define so clearly the conditions of survival of the larger forms, but the evidence at hand suggests that the failure to utilize washed living food as shown by *C. colpoda* is perhaps a basic characteristic. In addition, the factor of delicacy enters in to greatly restrict the possible media to which the larger forms can adjust themselves.

A holotrich, *Urocentrum turbo*, a hypotrich, *Pleurotricha lanceo-*

lata, and a heterotrich, *Bursaria truncatella*, may be considered. *Urocentrum* is interesting because it suggests a possible separation of the nutritive factor from that of delicacy. The author was fortunate in obtaining in pure culture the specific bacterium, *Achromobacter rodonatum*, supporting an excellent culture of these extremely active animals. *Urocentrum* will disintegrate, usually within a few hours, in Peters' medium with or without the bacterium in suspension. Apparently no bacteria are ingested. In the same medium and the same bacteria, growth is excellent if a rather active level of bacterial metabolism is maintained through the addition of wheat. *Urocentrum* is not much larger than *C. colpoda*, and will tolerate marked bacterial overgrowth and shifts in hydrogen ion concentration.

The fact that this disintegration is not referable to an unsatisfactory salt system is demonstrated by the extensive work of Penn (1933) on *Pleurotricha*. Experimenting upon spring waters and thirteen physiological media, he found that disintegration occurred in all of them, but that Peters' medium was somewhat less damaging than any of the others.

Bursaria truncatella, which has been worked on by both Dr. Penn and myself, is a very large and correspondingly delicate ciliate. The normal food consists of other protozoa, such as *Colpidium* and *Chilomonas*. Disintegration, in the presence or absence of carefully washed food, proceeds quite as rapidly as in the case of *Urocentrum*. However, tolerance to bacterial overgrowth is greatly reduced and normal survival appears to be conditioned upon a clear system resulting from a previous active bacterial metabolism in vessels which have not been disturbed.

My studies on *Stentor coeruleus* (1932*b*) and the measurements of Specht (1933) on the large *Spirostomum ambiguum*, show that these ciliates are independent of the oxygen tension over a very wide range. *Stentor coeruleus* is aberrant in that it does not disintegrate promptly when placed in physiological medium, but rather undergoes very slow degeneration. Under the most carefully prepared conditions it has been possible to maintain the division rate reported in my 1932*a* paper for one month, after which ingestion of food is curtailed and the size of the organism gradually diminishes. This is described in my thesis (1932*b*).

Colpidium colpoda thus occupies a somewhat intermediate position in its nutritive requirements between the very small and the very large ciliates.

The nature of the chemical consequences of bacterial metabolism, which appear to be crucial in the survival of these larger ciliate protozoa, is at present quite obscure.

SUMMARY

1. *Colpidium colpoda* will not grow on dead bacteria in non-nutrient salt solution (Peters' medium).

2. Growth on carefully washed living bacteria in Peters' medium will continue for a few generations only.

3. Unwashed, seven of the nine pure strains of bacteria will support rather irregular growth.

4. A bacterium (6—not identified) which exhibited marked activity after introduction into Peters' medium, gave the best growth (3.17 divisions per day).

5. Growth on bacteria in relatively simple nutrient, such as uric acid plus glycerol (for *Aerobacter aerogenes*), may be continued in subcultures only if the bacteria are inoculated several days before the ciliates.

6. Culture on bacteria in complex nutrient such as peptone-dextrose gave excellent growth with unexpected uniformity in subculture over the periods maintained: 8–22 months.

7. The predominant bacteria isolated from good cultures of ciliates were successful. Accompanying bacteria from such cultures were more or less unsatisfactory.

8. Media of complex nutrient (*a*) containing nutrient particles, including dead bacteria, in suspension, and (*b*) showing a range of oxygen tensions and of negative reduction potentials, failed uniformly to support *Colpidium* in pure culture.

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